BIOCHEMICAL AND MORPHOLOGICAL SYSTEMATICS OF THE SOUTHERN AFRICAN GASTROPOD GENERA *BURNUPENA* (BUCCINIDAE) AND *OXYSTELE* (TROCHIDAE)

by

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DECLARATION

This thesis documents original research, carried out in the Zoology Department, University of Cape Town. It has not been submitted in whole or in part for a degree at any other university. All assistance that I have received has been fully acknowledged.

Y. L. Dempster

15/8/95

Date

To my family, Allan, Nicole and the girls for their love and support.

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ABSTRACT

Dempster, Y. L. 1995. Biochemical and morphological systematics of the Southern African gastropod genera *Burnupena* (Buccinidae) and *Oxystele* (Trochidae). PhD Thesis, Dept. of Zoology, University of Cape Town, Rondebosch 7700, South Africa.

Gastropods of the genus *Burnupena* are very common rocky-shore whelks endemic to the Southern African region. However, despite recent revisions of the genus, difficulties in identification persist, due mainly to the occurrence of phenotypic intermediates between sympatric species. Samples of *Burnupena* were collected from 17 sites along the coast of South Africa. Variation in shell morphology was examined using multivariate analyses. The results revealed that the degree of variation within species is very high, and that shell form and colour can be influenced by local environmental conditions. Nevertheless, most populations of the individual species clustered together. Morphological differences between species were small, although distinctions could be discerned, with not all species being confused with all other species. However, on the basis of morphology, there will always be a few individuals which will remain difficult to identify.

Examination of the radulae from all of the species of *Burnupena* using both light and electron microscopy showed that the radula is of little or no practical value in distinguishing between species. Whilst some differences could be detected in some characters, these were not consistent, either within or between species. However, the radula is diagnostic at the generic level.

The populations of *Burnupena* were analysed for allozyme variation at 25 loci. Variation within species was high, and in all of the species, significant population differentiation was observed. A significant correlation between genetic and geographic distance was found. Nei's genetic distances within species were low (means per species ranging from 0.01 to 0.05), and the populations of each species clustered together. Unlike their morphological differences, the species

were mostly well differentiated genetically, with Nei's distances between species ranging from 0.1 to 1.45. Compared to the other species, *B. cincta* and *B. lagenaria* were relatively little differentiated from each other. *B. catarrhacta* was strongly differentiated from all of the other species. Comparisons between sympatric populations suggested complete reproductive isolation between the species.

The morphological and electrophoretic data supported the reduction of *B. limbosa* to a subspecies of *B. cincta*. Two new species were distinguished. One of these had been previously confused with a number of other species. The second cryptic species was only discerned during the electrophoretic analyses.

On the basis of the taxa recognised from the morphological and genetic studies, the taxonomy of the genus *Burnupena* Iredale, 1918 is reviewed, and each species is described and illustrated.

Electrophoretic and morphometric characters were also used to formulate phylogenetic hypotheses for the relationships within the genus *Burnupena*. A species belonging to a closely related genus, *Afrocominella capensis*, was used as the outgroup taxon. The results suggest that the genus *Burnupena* is monophyletic, and that *B. catarrhacta* is the most primitive species, and a sister species to all of the other species of *Burnupena*, which form a monophyletic clade. *B. cincta cincta* and *B. c. limbosa* were shown to be very closely related taxa, supporting their proposed subspecific status. Cladograms showed that *B. lagenaria* and the two subspecies of *B. cincta* constitute a distinct and closely related cluster. *B. papyracea* and *B. pubescens* are also closely related and, together with *B. c. cincta*, *B. c. limbosa* and *B. lagenaria*, form a monophyletic group. The placements of the two new species, *B.* sp. A and *B.* sp. B, are not clear.

Employing the same morphometric and genetic approaches, an analysis was made of *Oxystele variegata* (Anton, 1838), one of the commonest intertidal topshells on the coast of South Africa. Shell colour patterns, scanning electron micrographs of the central tooth of the radula, and

six enzyme loci were examined for samples collected from 14 sites around the South African coast. The results revealed that the so-called *O. variegata* actually consists of two sympatric species, *O. impervia* (Menke, 1843) and *O. variegata* (Anton, 1838). Both species exhibit an immense variety of shell colours and pattern, but consistent differences were observed. The shell of *O. impervia* has a pattern of dark red, orange, yellow or brown maculations, on a ground colour that is red, orange, yellow or brown, respectively. That of *O. variegata* has a pattern of red maculations or lines on a ground colour that is either off-white, or greenish-grey. The radulae of both species were also consistently different: the central tooth of the radula of *O. impervia* usually has a shallow base and a cusp that is slightly indented, whereas that of *O. variegata* usually has a deep base, and a well indented cusp. Further differences between the species were revealed by electrophoretic studies. Of six loci examined, two were found to be taxonomically diagnostic. Another two had at least one species-specific allele at a frequency of 0.1 or greater, and significant heterogeneity in allelic frequency between the two species was found in the four non-diagnostic loci. The differences in shell colour patterns, radular tooth structure and enzyme mobilities support the conclusion that *O. impervia* and *O. variegata* are separate but closely related species, with similar geographic distributions and an overlapping zonation.

GENERAL INTRODUCTION

Traditionally, molluscs have been identified and classified based on morphometric characters, usually their shell form, and to a lesser extent their radulae and internal anatomy. However, it is well known that shell form is sensitive to a number of environmental influences, and that plasticity of shell morphology can result in considerable variation within species along environmental and geographic gradients (e.g. Phillips *et al.*, 1973; Kitching, 1976; Crothers, 1983; Appleton & Palmer 1988; Palmer, 1990; Boulding *et al.*, 1993). On the other hand, environmental influences can result in convergence in shell shape among species (e.g. Ponder, 1973; Harasewych, 1984; Janson, 1985; Emberton, 1991; Kool, 1993), and a number of morphologically cryptic species have been identified (e.g. Mastro *et al.*, 1982; Ward & Janson, 1985; Palmer *et al.*, 1990; Staub *et al.*, 1990). Taxonomy based on shell morphology alone, is thus often insufficient, or worse, may be highly suspect (Kool, 1993).

In the last twenty to thirty years several molecular techniques have been increasingly employed, often in conjunction with traditional morphological studies, as a tool in systematic studies. The early applications of molecules to such studies involved the use of proteins, in particular, enzyme electrophoresis, a technique that has been used to investigate many problems, including genetic variability in natural populations, gene flow, hybridization, recognition of species boundaries and phylogenetic relationships (Murphy *et al.*, 1990). Moritz and Hillis (1990) reported that enzyme electrophoresis was the most widely used approach in molecular systematics, and a number of reviews on this topic have been published (e.g. Avise, 1975; Ferguson, 1980; Thorpe, 1982; Buth, 1984; Hillis & Moritz, 1990; Thorpe & Solé-Cava; 1994). With advances in technology, techniques involving the use of DNA and RNA have been widely employed since the late 1970's. These include approaches such as restriction site analysis, DNA hybridization and sequencing (Moritz & Hillis, 1990). Although enzyme electrophoresis and restriction site analysis of DNA can and have been applied to address many of the same problems (Hillis & Moritz, 1990), the use of protein electrophoresis remains among the cheapest and most cost-effective methods (Grant *et al.*,

1988; Murphy *et al.*, 1990), and is likely to play an important role in molluscan systematics for many years (Emberton, 1994).

The genus *Burnupena* (Neogastropoda: Buccinidae) is endemic to Southern Africa, and contains some of the commonest neogastropods inhabiting this coastline (Kilburn & Rippey, 1982). Six species are currently recognised, namely *B. catarrhacta* (Gmelin, 1791), *B. cincta* (Röding, 1798), *B. lagenaria* (Lamarck, 1822), *B. limbosa* (Lamarck, 1822), *B. papyracea* (Bruguière, 1789) and *B. pubescens* (Küster, 1858). Of these, three have been known by other names, two of which have been altered only recently. *B. cincta* was known as *B. porcata*, but this name was replaced by Iredale (1918) when he erected the genus *Burnupena*. *B. pubescens* was known as *B. tigrina* until it was changed by Kilburn (1972), and even more recently, *B. delalandii* was replaced by *B. catarrhacta* (Kilburn & Rippey, 1982).

There have been two relatively recent revisions of the genus (Orr, 1956; Barnard, 1959), but difficulties in identification persist, due mainly to the occurrence of phenotypic intermediates between sympatric species (Kilburn, 1972, Kilburn & Rippey, 1982). Stephenson (1948) frequently encountered specimens which he could not assign to one or another species as they were intermediate in form, and he suggested that they could be hybrids. In her revision, Orr (1956) regarded only two species as valid, preferring instead to reduce four of the species to subspecies, and to synonymize others. Although Barnard (1959) recognised six species in his revision, he noted that some specimens were impossible to identify with certainty. Most of the difficulties that have arisen centre around particular combinations, with the most confusing species pairs being *B. cincta* and *B. lagenaria*, and *B. lagenaria* and *B. catarrhacta*. Further confusion arose early in my study, with the collection of two populations of snails on the west coast which did not fully conform with any of the species described. These snails were however, identical to specimens in the reference collection at the South African Museum (A36383), which had been identified by K. H. Barnard as *B. limbosa*. These snails were subsequently found to be a undescribed species.

Although Kilburn and Rippey (1982) recognised six species, they described the genus as "a headache to collectors and an embarrassment to malacologists, who are quite unable to agree on the number of species, their limits or their relationships!".

Apart from *B. pubescens*, all of the other species are common to abundant around the coastline of Southern Africa (Stephenson *et al.*, 1940; Stephenson, 1944). In their section on marine provinces, Kilburn & Rippey (1982) list three species of *Burnupena*, namely *B. catarrhacta*, *B. limbosa* and *B. papyracea*, amongst those species characteristic of the Namaqua Province. They note that in the Algoa Province, these species are replaced by *B. lagenaria* and *B. cincta*. Members of this genus are therefore of ecological and biogeographic interest, and a revision of the genus with the intention of resolving the taxonomic problems is long overdue.

In view of the limitations of traditional morphology to resolve taxonomic issues, my thesis combines both morphological and biochemical (enzyme electrophoresis) methodologies in an attempt to determine the number of species within the genus *Burnupena*, and to discover their evolutionary affinities. To achieve my goal, it was first necessary to examine levels of variation within populations and within species. A number of studies has provided evidence to support the view that marine molluscs with limited dispersal, and hence gene flow, show greater population differentiation than those with broad dispersal (e.g. Ward & Warwick, 1980; Burton, 1983; Janson & Ward, 1984; Grant & Utter, 1987; Day, 1990). Given that *Burnupena* species do not have a pelagic larval stage (Bokenham *et al.*, 1938), one might predict that levels of intraspecific variation, both morphological and electrophoretic, will be high, and that conspecific populations in close proximity will be less differentiated than those that are geographically well separated.

Armed with a knowledge of the degree of variation within species, it was then possible to assess the differentiation between species and to address a number of questions. Firstly, can the species be distinguished by shell characters alone? Secondly, are the current morphologically-based species genetically distinct? The effect of environmental factors on shell form can be examined by comparing populations of different species which occur in sympatry, with populations of the same

species which are well separated geographically. If shell morphology is influenced largely by the environment, then one might expect to find sympatric species which are genetically distinct, but morphologically similar, and allopatric conspecific populations which are genetically similar but morphologically different. Thirdly, how are the species related? Are those species which are difficult to differentiate morphologically, or are electrophoretically similar, the most closely related, and do phylogenies based on morphological characters concur with those based on electrophoretic data?

A number of studies, both morphological and electrophoretic, have been made on species belonging to the genus *Nucella* (e.g., Grant & Utter, 1987; Appleton & Palmer, 1988; Day & Bayne, 1988; Day, 1990; Palmer, 1990; Palmer *et al.*, 1990; Day *et al.*, 1993; Gibbs, 1993), which is a member of the Muricidae, a family that is closely related to the Buccinidae (Ponder, 1973; Kool, 1993). Furthermore, species of *Nucella*, like *Burnupena*, do not have a pelagic larval stage, and therefore, the results of my studies of *Burnupena* can be closely compared with those of the *Nucella* studies.

The methods that were used for *Burnupena* can be applied to other species. A case in point presented itself during the earlier part of my studies on *Burnupena*, when an examination of *Oxystele variegata* snails in the Cape Peninsula by Joseph Heller, revealed colour differences in some of the shells, indicating the possible presence of a cryptic species. A study of this species was conducted, which has since been published (Heller & Dempster, 1991), and is presented in Chapter 6. The shell colour and taxonomic sections were done by J. Heller, the electron microscopy of the radulae was done jointly, and the electrophoretic analyses were performed by myself.

The thesis consists of seven Chapters, the outlines of which are given below:

In Chapter 1, I examine the morphological variation both within and between the species of *Burnupena*. Individuals from 29 populations, representing eight species, were collected along the coast of South Africa. I use both quantitative and qualitative data obtained from the shells, which were analysed using standard univariate and multivariate methods of analysis. Initially I examine the genus as a whole to develop an understanding of the overall picture. I then consider each species separately, examining both within-population, and within-species levels of variation. Finally I discuss the differentiation between the species.

In Chapter 2, I examine the radulae of all species using both light and scanning electron microscopy to determine the value of radular structure in distinguishing the species. I also compare the radulae of *Burnupena* and those of a closely related genus, *Afrocominella*.

In Chapter 3, I investigate the population structure and species boundaries in *Burnupena* based on biochemical data. Individuals from 38 populations were electrophoresed using established protein gel electrophoretic techniques. Initially I explore and discuss the levels of genetic variability within each of the species. This is followed by considering intraspecific differentiation and population structure. Finally I examine the genetic differentiation between the species and discuss the systematics of the genus.

In Chapter 4, I present a taxonomic review of the genus *Burnupena*, based on the results from the first three Chapters, as well as examination of specimens from a number of museum collections. I recognise five of the current species, together with one subspecies and two new species. For each of the species, I give details of their description, diagnostic features and distribution, together with remarks regarding their taxonomic history. I provide a key to the species and summarize the results of this and the previous three Chapters.

In Chapter 5, I formulate a phylogenetic hypothesis for the species of *Burnupena*. I use both morphological and allozyme data for the construction of evolutionary trees, with the emphasis on the latter data. Both phenetic and cladistic approaches are employed. The resulting trees are compared and discussed.

In Chapter 6, I present morphological and electrophoretic evidence which reveals that *Oxystele variegata* comprises two cryptic species. Shell colour variation, differences in the radula, and differences in enzyme mobility are examined, and in the light of these results, two species are described, and a key for their identification provided. As acknowledged above, this section of my thesis was done in conjunction with J. Heller.

I close my thesis with the seventh Chapter, in which I synthesize the results of the previous Chapters. I assess levels of variation within species, with reference to environmental influences and mode of reproduction, and discuss levels of differentiation between species. I also argue the relative merits of different methodological approaches to the study of systematics in marine gastropods.

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Chapter 1

Multivariate analysis of shell morphology in Burnupena

INTRODUCTION

Gastropods of the genus *Burnupena* comprise a group of very common rocky shore whelks endemic to the Southern African region. However, the distinction between the different species is not always clear, leading to difficulties in identification, due to the frequent occurrence of intergrading forms (Barnard, 1959). In a taxonomic revision of the group, Orr (1956) recognised two species, *B. papyracea* (Bruguière, 1789) and *B. delalandii* (Kiener, 1834), and four subspecies of *B. papyracea*, namely *B. p. papyracea* (Bruguière, 1789), *B. p. cincta* (Röding, 1798), *B. p. lagenaria* (Lamarck, 1822) and *B. p. tigrina* (Kiener, 1834). Barnard (1959) and Kilburn and Rippey (1982) recognised six species, elevating Orr's four subspecies, as well as one of her synonyms of *B. papyracea*, namely *B. limbosa* (Lamarck, 1822), to the specific level.

There have been two name changes in recent years. *B. tigrina* (Kiener, 1834) was renamed by Kilburn (1972) as *B. pubescens* (Küster, 1858) since the name *tigrina* had been used earlier by Gmelin (1791) to describe a completely different species. *B. delalandii* (Kiener, 1834) was changed by Kilburn and Rippey (1982) to *B. catarrhacta* (Gmelin, 1791) since the latter name had precedence.

There is little problem separating most pairs of *Burnupena* species morphologically. However, considerable difficulties have arisen in distinguishing particular combinations. In regions of geographic overlap, Stephenson (1948) "not infrequently encountered" specimens of either *B. cincta* or *B. lagenaria*, which he could not assign to one or other of these species as "they seem to be genuine intermediates, conceivably hybrids". He found the same problem with specimens of *B. cincta* and *B. catarrhacta*. He also found that two of the West coast species, *B. papyracea* and *B. limbosa*, while usually distinguishable, were "sometimes rather similar". In her paper, Orr (1956) found that intermediates between the latter two species were common and regarded *B. limbosa* a synonym of *B. papyracea papyracea*. Barnard (1959) noted that "some examples are impossible to assign definitely to one species or another" and that "the two most confusing pairs are *cincta* -

lagenaria and *limbosa - catarrhacta*". Kilburn and Rippey (1982) noted that *B. cincta* intergrades with both *B. lagenaria* and *B. pubescens*, and that in the Western Cape *B. lagenaria* is difficult to differentiate from *B. catarrhacta*. In a preliminary study of the genus (Dempster, 1986) I encountered difficulties in distinguishing between *B. limbosa*, *B. lagenaria* and *B. catarrhacta* from the West Coast. In the current more detailed study, problems of specific assignment were also encountered between the following combinations: *B. papyracea* and *B. pubescens*; the latter and a previously undescribed species; *B. cincta* and *B. limbosa* on the West coast; and between *B. cincta*, *B. lagenaria* and *B. catarrhacta* at Kommetjie. It is evident from this and from the earlier literature (e.g. Stephenson *et al.*, 1940) that there is a lot of confusion between *B. catarrhacta* and *B. lagenaria* on the West coast. In some of the literature (Stephenson, 1944; Stephenson, 1948; Orr, 1956) the range of *B. lagenaria* is given as not extending onto the West coast. Barnard (1959) gives the range from False Bay to Natal, but notes the existence of specimens from a few localities on the West coast from as far north as Walvis Bay in Namibia. Kilburn and Rippey (1982) give the range as Natal to Saldanha and probably extending up to Namibia. In my study it was found to be much more common than *B. catarrhacta* on the West coast. All of the above references agree on the range of *B. catarrhacta*: West coast extending round to Hermanus in the Western Overlap. However, many of the specimens located in the South African Museum, which would have been available to Barnard and Stephenson, were identified as *B. catarrhacta* from the West coast, although I found them to be *B. lagenaria*.

Variations in molluscan shell form have been shown to be sensitive to environmental factors such as predation, temperature, salinity, wave action, diet, desiccation and substratum, as well as population density (Moore, 1936; Phillips *et al.*, 1973; Spight, 1973; Kitching, 1976; Smith, 1981; Currey & Hughes, 1982; Crothers, 1983; Kemp & Bertness, 1984; Chow, 1987; Appleton & Palmer 1988; Thomas & Himmelman, 1988; Boulding, 1990; Munksgaard, 1990; Palmer, 1990; Boulding *et al.*, 1993). Hence, morphological differences do not necessarily indicate species differences (Gould *et al.*, 1975; Raffaelli, 1979; Kemp & Bertness, 1984; Palmer, 1985), and many species of marine gastropods are known for their highly variable shells. On the other hand, lack of morphological differences do not always indicate that there are no species differences, and a

number of cryptic gastropod species have been identified (Chambers, 1978; Mastro et *al.*, 1982; Janson, 1985; Ward & Janson, 1985; Palmer et *al.*, 1990; Staub et *al.*, 1990).

Multivariate methods for the analysis of morphometric data, such as discriminant analysis and principal component analysis, have been widely used for the morphological separation of different groups of marine molluscs (Phillips et *al.*, 1973; Murray, 1982; Janson & Sundberg, 1983; Ward & Janson, 1985; Chow, 1987; Tissot, 1988; Grahame et *al.*, 1990; Johannesson & Johannesson, 1990; Boulding et *al.*, 1993). With these methods the information contained in a number of morphometric variables can be reduced to a few composite variables which summarize most of the information and differentiate between the groups better than any individual morphometric variable (Reyment et *al.*, 1984).

All of my initial species assignments were based on shell characters and followed the descriptions in Kilburn and Rippey (1982). For the majority of the specimens these identifications were confirmed by the allozyme electrophoretic analyses. However, in a few cases it was found that the initial identification was incorrect, and for these animals (noted in the results), the specific assignment was modified accordingly, prior to morphological analysis by multivariate methods.

KEY QUESTIONS

Given that shell form can be influenced by environmental factors, it is important to assess the amount of variation within populations and within species to put into perspective the differences between species. The purpose of this section of my study was to examine the morphological variation in a number of shell characters, and to use these data to answer the following questions:

(a) How much morphological variation exists within local populations?

(b) How much intraspecific variation is there, and are some species more variable than others?

(c) How much morphological differentiation exists between different species?

(d) Are morphological differences between different species greater in regions where they co-occur?

(e) Can shell characters be used to distinguish the members of the genus?

MATERIALS AND METHODS

Samples of adult *Burnupena* were collected from 14 sites along the coast of South Africa between January 1987 and February 1993 (Fig. 1). The South African coastline has been divided into a number of biogeographical provinces (see Fig. 1) which correspond to changes in the ocean currents. The regions used in this study follow those described by Stephenson (1944), who recognised three major faunal provinces with zones of overlap between them. The West Coast province (WC) is bathed by the cold, northerly-moving Benguela Current, and includes the stretch of coastline from Namibia to beyond Cape Point. Emanuel *et al.* (1992) have subsequently shown that this region is separable into two major provinces, divisible at Luderitz. The East Coast (EC) province is bathed by the warm, southerly-flowing Agulhas Current, and stretches approximately from Southern Mozambique to Transkei. The South Coast faunal province (SC) lies between these two provinces, stretching from Cape Point to Transkei. The current bathing this stretch of coastline is cooler than the Agulhas Current (which in this region has swung away from the shore). There are however, zones of overlap between these provinces. The Western Overlap (WO) lies between Kommetjie and Cape Agulhas, and includes False Bay. This zone contains a mixture of water from both the Benguela and Agulhas Currents. Emanuel *et al.* (1992) combine the Western Overlap with the South Coast region, which they refer to as the Warm Temperate South Coast. Stephenson's regions have been followed in this study since the geographic distances between the localities sampled in the Western Overlap and those in the South Coast region were great.

In total, 29 populations were sampled, since at most sites more than one species was found. Snails were collected at random, and where possible, only the largest specimens were retained. For three of the species, namely *B. papyracea*, *B. pubescens* and *B.* sp. B, all populations were collected subtidally, whilst all populations of *B. lagenaria* and *B. catarrhacta* were found in the intertidal zone. For the other three species, *B. cincta*, *B. limbosa* and *B.* sp. A, some populations were collected subtidally and others intertidally. All of the sampling localities were exposed to heavy wave action. Details of the localities, zonation, abbreviations, and the number of animals collected for each population are given in Table 1. The population codes used throughout consist of the species and site abbreviations, separated by a dash (e.g. the sample of *B. papyracea* collected at Blouberg is referred to as PAP-BB).

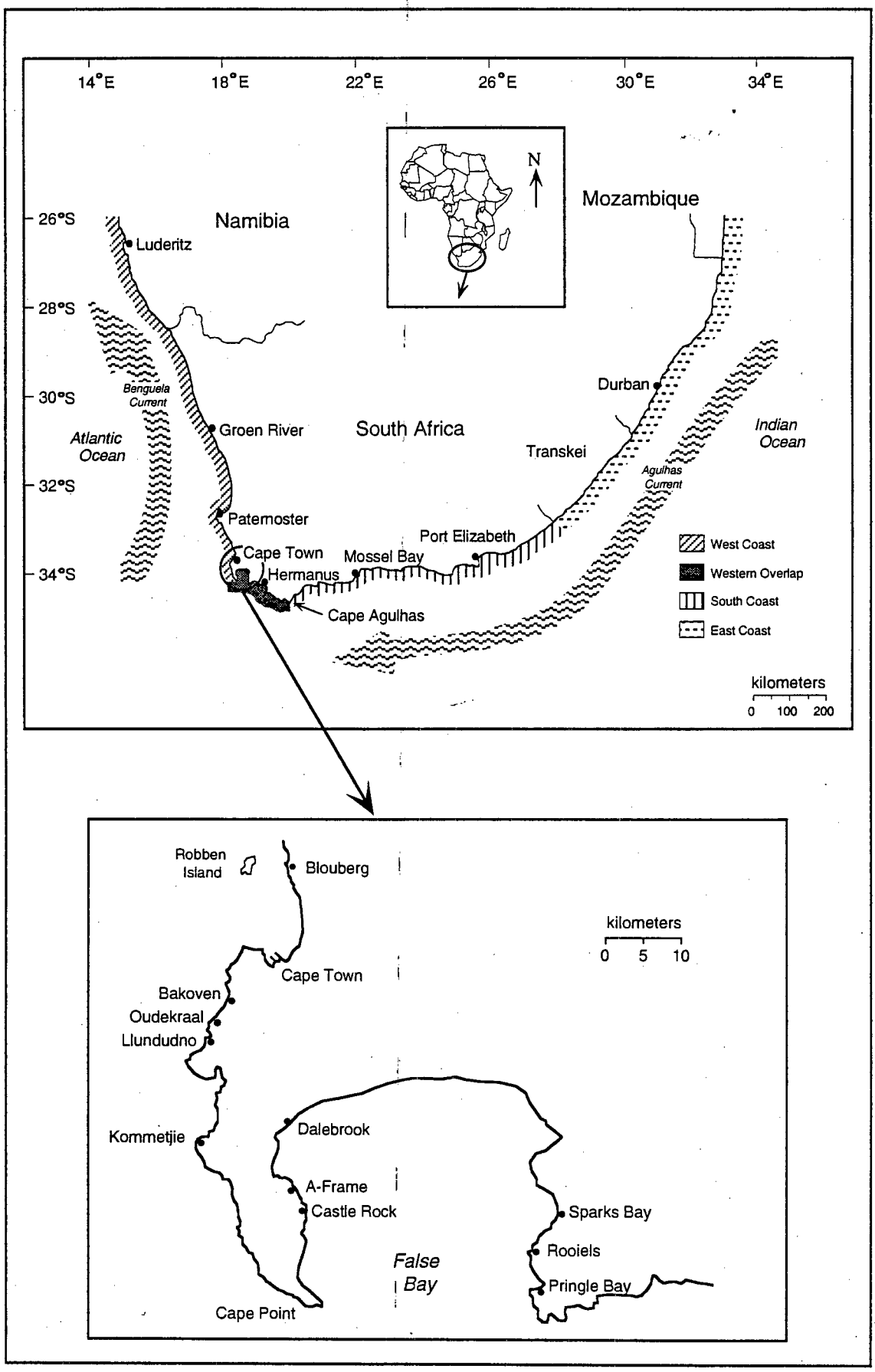


Figure 1. Map of Southern Africa indicating the sampling localities and other names mentioned in the text. The shaded areas show the geographic regions and the direction of the Benguela and Agulhas currents are indicated.

Table 1. Locality data, abbreviations used and sample size for the populations of *Burnupena* collected.

Region	Locality Site	(abbrev.)	km[4]	Species collected[1] PAP	B[2]	PUB	CIN	LIM	A	LAG	CAT
West Coast (WC)	Groen River	(GR)							23	37	
	Blouberg	(BB)	422	31				18		14	
	Bakoven	(BO)	28	50					36[5]		
	Oudekraal	(OK)	4					29[3,5]			
	Kommetjie	(KM)	30					32		29	18
Western Overlap (WO)	Castle Rock	(CR)	48	42	1	33	21[5]				
	A-Frame	(AF)	4	58[3]	16	30[3]	57[3,5]				
	Dalebrook	(DK)	13				62[3]			33	22[3]
	Sparks Bay	(SB)	56							19	
	Rooiels	(RE)	7		2	7					
	Hermanus	(HM)	57							17	
South Coast (SC)	Mossel Bay	(MB)	170				45			39	
	Port Elizabeth	(PE)	380			5	22				
East Coast (EC)	Durban	(DN)	900							27	

[1] Species: PAP = *B. papyracea*; B = *B. sp. B*; PUB = *B. pubescens*; CIN = *B. cincta*; LIM = *B. limbosa*; A = *B.* sp. A; Lag = *B. lagenaria*; CAT = *B. catarrhacta*.

[2] The 16 specimens from AF were collected on 3 separate trips (within 3 months), but there was no difference between the means for shell length. The specimens collected at CR (1) and RE (2) were smaller and were not used in the main analysis.

[3] Collections were made on two different dates (less than a year apart) but there were no differences between the means of the 2 samples, which have been pooled for analyses.

[4] Distance in km between successive sites

[5] Populations of *B. cincta*, *B. limbosa* and *B.* sp. A collected subtidally. The remaining populations of these three species were collected intertidally. All populations of *B. papyracea*, *B. pubescens* and *B.* sp. B were collected subtidally. All populations of *B. lagenaria* and *B. catarrhacta* were collected intertidally.

The initial species assignments were based on shell characters and for the most part followed the descriptions given in Kilburn and Rippey (1982), although two additional forms were found. Two populations sampled on the West Coast did not conform to any of Kilburn & Rippey's species descriptions, but closely resembled a specimen figured by Orr (1956, Plate 19, Fig. 4) which she identified as *B. papyracea papyracea*. However, I easily recognise and distinguish this form from *B. papyracea* and will hereafter refer to it as *B.* sp. A. Both this species and *B. papyracea* were collected subtidally at Bakoven; this sympatry added weight to the conclusion that they were not the same species. Furthermore, animals from all of the populations of *B. papyracea* were covered by a bryozoan (although dead specimens often loose this covering), whilst the animals from the two *B.* sp. A populations never carried it.

The second form which did not correspond to any previous description was found in very low numbers at three sites in False Bay. It was found together with *B. papyracea* and *B. pubescens*, and although the shell appeared to be slightly different to that of *B. pubescens*, the first few individuals collected were initially assigned to this species. However, the results of the allozyme electrophoretic analysis indicated that this form could be distinguished from *B. pubescens* (see Chapter 3), and these individuals, plus those subsequently collected, were assigned to a new species, hereafter referred to as *B.* sp. B.

For reasons that are argued below (page 61) I believe that *B. limbosa* should be reduced to a subspecies of *B. cincta*. I therefore use the terms *B. cincta cincta* and *B. cincta limbosa* (and the abbreviations CIN and LIM) to distinguish them.

The shells of three species, namely, *B. papyracea*, *B. pubescens* and the new species, *B.* sp. B, were covered by a bryozoan which had to be removed before the snail could be identified. Although this bryozoan outwardly looks to be the same on each of the species, and these co-occur, it is only from specimens of *B. papyracea* that the bryozoan has been identified with certainty as *Alcyonidium nodosum* (O' Donohue & De Watteville 1944).

In most cases individuals that were used for the morphometric analyses were also used for the electrophoretic analyses. Measurements were taken prior to dissection of the animals. Ten shell and opercular measurements were made to the nearest 0.01 mm using digital calipers (Fig. 2a). The length measurements, (shell, aperture and operculum - SL, AL, OL), were taken as the maximum length; width measurements (shell, aperture and operculum - SW, AW, OW) were the maximum widths perpendicular to the respective lengths. These six measurements, together with spire (SP) and shoulder heights (HT), are referred to as shell size variables. Two measurements were taken to give an indication of the thickness of the shell. The first one (T1), was taken at the outer edge of the aperture about 1mm in from the edge, and the other, (T2), taken at few mm in from the edge of the outer lip, approximately level with the dorsal edge of the siphonal notch (see Fig. 2b). These latter two variables are referred to as shell thickness variables.

Immediately prior to dissection, the snails were pressed into their shells and blotted to remove as much extravisceral water as possible. The snails were then weighed (total wet weight), after which the animals were extracted whole from their shells and the wet body weight (BWT) was recorded to the nearest 0.1 g. The shell weight (SWT) was obtained by subtracting the body weight from the total weight. These two weights are also referred to as size variables. After the removal of the animal from its shell, the sex was determined whenever possible. If the penis, vas deferens or testis, or any combination of these was observed, the individual was scored as a male. If none of these was seen, and there was an obvious albumin gland, the individual was scored as female. If none of these characters was clearly obvious (usually in smaller individuals), then the individual was regarded as a juvenile and was not used in the data analysis.

The following six qualitative shell characters were examined and graded or coded (see Table 2 for description of codes). Two variables were used to describe the pattern of spiral shell ribbing (RIB1 and RIB2). The first, indicating the strength of the ribs, had four categories ranging from smooth to strongly ribbed. The second indicated the number of ribs on the body whorl and also had four categories, which ranged from none to many. The degree of flattening of the posterior end of the outer lip of the aperture against the body whorl at the point of adhesion (Fig. 2), or the

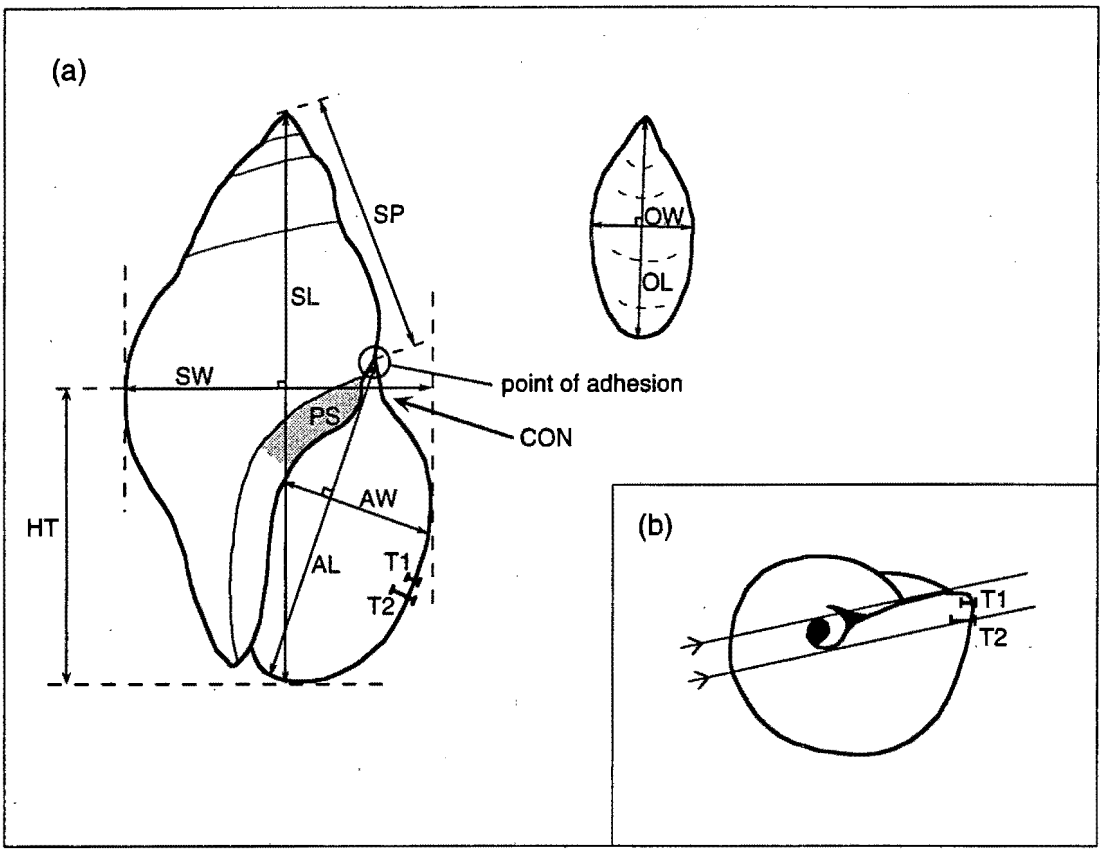


Figure 2. Illustration of the shell and operculum showing the dimensions measured. (a) shows the apertural view and operculum, and (b) shows the view from the basal end to indicate the approximate depth of the shell thickness measurements. Dimensions are as follows: SL = maximum shell length; SW = maximum shell width perpendicular to shell length; SP = spire height; AL = maximum aperture length; AW = maximum aperture width perpendicular to aperture height; HT = height of the body whorl at the maximum width, or shoulder height; T1 = shell thickness at the edge of the outer lip (about 1mm down), and measured towards the middle of the outer lip of the aperture; T2 = shell thickness taken a few mm below the edge of the outer lip (approximately level with the dorsal side of the siphonal notch), and also measured towards the middle of the outer lip of the aperture; OL = maximum operculum length; OW = maximum operculum width perpendicular to operculum length. The positions of the constriction (CON) and the parietal scar (PS) are also indicated.

Table 2. List of qualitative shell characters and their states.

Character	States
rib strength (RIB1)	1: smooth 2: smooth upper half; weak lower half 3: weak 4: strong
no. of ribs on body whorl (RIB2)	1: none 2: few (4 - 9) 3: intermediate (10 - 14) 4: many (> 14)
constriction (CON)	1: none 2: slight 3: moderate 4: marked
aperture sculpture (AS)	1: smooth 2: plicate at edge of outer lip only 3: plicate internally
parietal scar (PS)	1: none 2: pale 3: dark
spire angle (SA)	1: < 40° 2: 40° to 50° 3: 50° to 60° 4: 60° to 70° 5: 70° to 80° 6: > 80°

constriction of the outer lip (CON), which reflects the extent of a groove below the suture, was scored from 1 to 4. The amount of ridging on the inside of the aperture (aperture sculpture - AS) was coded from 1 to 3. The presence or absence, and intensity, of a brown scar on the parietal region (parietal scar - PS) was scored from 1 to 3. The spire angle (SA) was measured using a protractor and coded from 1 to 6, with a shell having a spire angle of less than 40^{o} coded as a 1, increasing at 10^{o} intervals to a rank of 6, representing a spire angle of greater than 80^{o}.

Correlations (calculated using Pearson product-moment correlation coefficient) between most of the quantitative variables within each species were high. Excluding shell thickness (T1 and T2), the minimum correlations ranged from 0.58 to 0.82, and the maximum from 0.96 to 0.99 within a species. The lower correlations were usually between spire height and one of the other variables, and if this variable was not considered, the minimums ranged from 0.68 to 0.86. In all of the species, there was a higher correlation between most of the variables and shell width rather than the length, although the height of the spire had a higher correlation with shell length. The correlations between T1 and T2 and the other variables were much lower, with the minimums ranging from 0.07 to 0.50, and the maximums from 0.66 to 0.84. These higher correlations were always either with each other, or with shell weight.

A set of ratio variables were calculated from the raw data. These served to indicate relative size, shell thickness and shape, thus making it easier to compare the populations of individuals with different mean sizes. Shell length was used as the standard denominator except for aperture and operculum width, for which the shell width was used as the denominator.

Several univariate and multivariate analyses were performed on the data. The programs 3D, 7D and 7M of the BMDP Statistical Software Package Inc. (version 1990) were used for t-tests, analysis of variance and Tukey's studentized range tests, and stepwise discriminant and canonical variate analyses. Principal components and cluster analyses were performed using the PRINCOM and CLUSTER procedures in SAS (SAS Institute Inc., Cary, NC, USA 1989). In many of the cases, the variances were not equal (Levene's test for equal variances). In these instances, the p-values used in the t-tests were those from the separate variance t-values, since these are more robust when

variances are not equal. If there were significant differences between males and females within a population for any variable, then the Tukey's test for comparisons between sites were done separately for each sex.

The non-parametric statistics of Welch and Brown-Forsythe were used for the analysis of variance. The 12 quantitative variables and six of the qualitative variables (the non-ordinal variables, shell and aperture colour, were excluded) were used in the discriminant analyses. The quantitative data were transformed to natural logarithms before the analysis to stabilise the variance. The "jackknife" option was used so that the classification functions computed to make group assignments for each case, were computed from all the data except the case being classified. A useful feature of discriminant analysis is that the classification function computed can be used to classify new cases whose group membership is unknown. In the plots of the canonical variables, closed convex polygons, or convex hulls, surrounding all of the individuals in each group, are used to show the distribution of the groups. The principal component analysis was based on the logged data and the correlation matrix of the 12 quantitative variables. The first eigenvector from this analysis can be interpreted as a size vector if all the elements are positive, of approximately equal size, and if they are equal to $(n)^{-1/2}$ where n is the number of characters (Jolicoeur, 1963). Variables which have low correlations with the remaining ones will dominate the first few vectors after the first principal component (Reyment *et. al.*, 1984). Both discriminant and principal component analyses were used in these analyses. The discriminant analysis requires that the individuals be placed into groups before the analysis, and tends to emphasise differences between groups by minimizing the variation within groups and maximizing the differences between the groups. Principal component analysis requires no *a priori* groups, and therefore does not emphasise group differences, but is used to investigate whether groups can be distinguished by the data. Another important difference between the two methods of analysis concerns the variables used. In discriminant analysis, only those variables which best discriminate between the groups are selected, so not all of the variables are necessarily used. In principal component analysis, all of the variables are used to produce fewer principal components which explain as much of the variance in the original variables as is possible (Afifi & Clark, 1990). If there was a significant difference between

the males and the females within a population for any variable, then the discriminant analysis was repeated using those males and females as separate groups. The cluster analyses were conducted on the group means using both quantitative and qualitative variables. These were standardized to a mean of zero and standard deviation of one. The Euclidean distance coefficient was used together with the unweighted pair-group method with arithmetic averages (UPGMA) clustering algorithm.

In three of the populations, LIM-OK, PUB-RE and PUB-PE, there were missing data for some of the variables. In the first of these, of the 29 individuals sampled, there were no data for height for 19 of the animals. In the multivariate analyses, it was decided to exclude this variable and use all the individuals in comparisons between populations of this species, but for analyses of differences between species, all 12 variables were used with the ten fully quantified individuals from this population. For the two *B. pubescens* populations, data were missing for four variables (body and shell weights, opercular length and width) for all animals sampled. Although the sample sizes of these two populations were small (n = 7, 5), it was felt that their inclusion was still useful in examining geographic differences. Therefore, for the discriminant and principal component analyses, only eight variables were used for this species. For between-species analyses, these two populations were excluded and all variables were used.

RESULTS

In the first section of the results, the genus as a whole is examined to develop an understanding of the overall picture and the problems that have arisen with identification based on morphological features. In the second section each species is considered separately in terms of within-population and within-species variation. The final section of the results deals with multivariate analyses performed on certain pairs of species, which have previously been difficult to distinguish, to assess their morphometric similarity. This section also examines the group placement of a number of specimens which could not be assigned to a species, as well as some of the type specimens.

VARIATION BETWEEN SPECIES

The means and standard deviations for each of the 18 variables for all eight species of *Burnupena* are given in Table 3. *B. cincta cincta* and *B. cincta limbosa* are the largest (mean shell length), followed by *B. papyracea*, whilst *B. lagenaria*, *B. pubescens* and *B. catarrhacta* are the smallest (Fig. 3). Analysis of variance of the quantitative variables indicated that for all of them, there was a significant difference ($p<0.001$) between the means of the species. Pairwise tests (28 comparisons using Tukey's range test) indicated that there were significant differences ($0.05>p>0.01$) for most of the shell size variables between all pairs of species, except between *B.* sp. B and *B.* sp. A (Table 4). Of the 27 comparisons with significant differences, in only five were there differences between three or fewer of the ten size variables. For the remaining comparisons, at least seven of the ten size variables showed significant differences. There were also significant differences ($0.05<p<0.01$) in shell thickness (T1 and/or T2) between 21 of the 28 pairs of species. *B.* sp. B was not significantly different to five of the other species for shell thickness (Table 4).

Table 3. Means and standard deviations of variables for all species of *Burnupena*. No standard deviations are given for the six qualitative variables.

Species[1]	PAP	B	PUB	CIN	LIM	A	LAG	CAT
shell length (sl)	41.93 8.00	39.11 3.34	31.11 4.43	46.87 7.15	45.29 6.45	37.03 4.26	31.43 4.71	28.83 3.43
shell width (sw)	21.85 3.97	20.14 1.51	16.25 2.22	24.87 3.47	25.03 3.13	22.71 2.71	18.79 2.64	15.28 1.62
spire height (sp)	21.06 4.66	18.64 2.09	15.06 2.37	21.69 4.04	20.74 3.81	15.77 2.40	12.53 2.54	13.60 1.83
aperture length (al)	23.10 4.07	22.42 1.72	17.48 2.21	27.78 3.57	27.36 3.15	24.12 2.56	21.16 2.71	16.94 1.85
aperture width (aw)	10.88 2.26	9.90 0.85	7.62 1.20	11.92 1.84	12.05 1.71	11.32 1.49	9.39 1.46	7.41 0.91
shoulder height (ht)	21.30 3.88	18.90 1.44	15.69 2.30	23.15 3.23	23.19 3.12	20.42 2.58	17.20 2.09	14.29 1.57
thickness 1 (t1)	0.56 0.22	0.53 0.17	0.40 0.15	0.55 0.22	0.55 0.26	0.76 0.40	0.50 0.19	0.34 0.11
thickness 2 (t2)	1.38 0.67	1.31 0.36	0.89 0.32	1.27 0.50	1.72 0.98	1.56 0.59	0.90 0.32	0.59 0.22
body weight (bwt)	3.41 1.67	2.39 0.65	1.21 0.55	4.02 1.76	3.70 1.61	3.13 1.19	1.72 0.83	0.95 0.36
shell weight (swt)	4.99 2.56	3.69 0.89	2.25 0.90	9.17 4.06	10.36 4.33	5.80 2.57	3.10 1.23	1.78 0.59
opercular length (ol)	15.02 3.03	14.69 1.53	10.34 1.40	17.77 2.88	17.00 2.58	14.41 2.07	12.11 1.65	8.83 1.05
opercular width (ow)	8.04 1.49	7.61 0.52	5.73 0.68	9.47 1.32	8.56 1.33	8.00 1.23	6.82 1.01	4.84 0.57
rib strength (rib1)	2.64	3.00	4.00	3.75	1.55	1.00	2.43	1.03
no. ribs (rib2)	2.11	4.00	3.00	2.00	1.55	1.00	1.94	1.03
constriction (con)	1.47	2.06	2.21	3.21	2.54	1.56	3.28	2.70
aperture sculpture (as)	2.76	2.94	2.74	2.01	2.99	2.93	2.21	2.18
parietal scar (ps)	1.75	1.81	1.82	1.89	2.06	2.80	2.75	2.98
spire angle (sa)	4.03	3.00	3.23	4.02	4.22	5.10	4.58	2.93
n =	181 ht:153 ol:149 ow:149	16	75 ht:65 bwt:62 swt:62 ol:50 ow:50	207 ht:190 ol:188 ow:188	79 ht:59	59	215 swt:210 ol:209 ow:210	40

[1]Key to species: PAP = *B. papyracea*; B = *B.* sp. B; PUB = *B. pubescens*; CIN = *B. cincta cincta*; LIM = *B. cincta limbosa*; A = *B.* sp. A; LAG = *B. lagenaria*; CAT = B. *catarrhacta*.

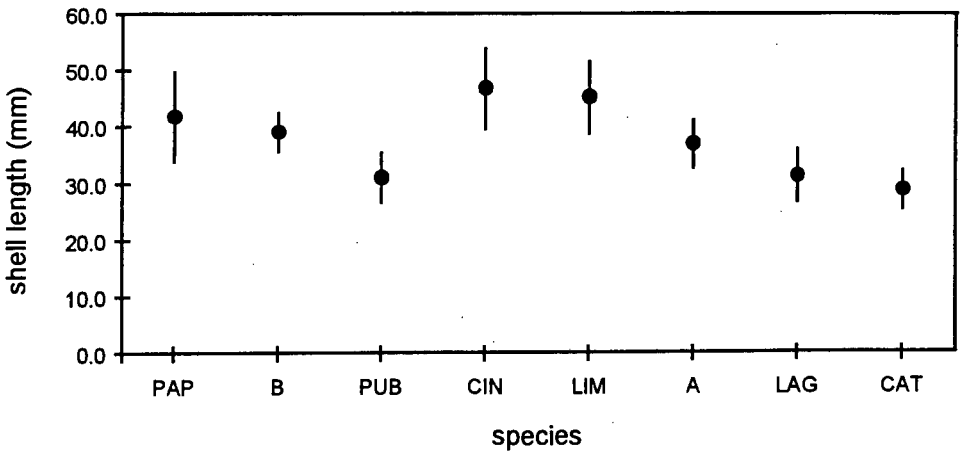


Figure 3. Mean shell length (± one standard deviation) for each of the species of *Burnupena*. See Table 3 for species abbreviations.

Table 4. Tukey's studentized range test for all eight species of *Bumupena*. The variable names in the cells indicate a significant difference between the two species for that variable.

	PAP						
B	s6						
PUB	s1-10 t1,2	B s1-7,9,10					
CIN	s1,2,4-10	s1-10	PUB s1-10 t1,2				
LIM	s1,2,4-6,8-10 t2	s1,2,4-9	s1-10 t1,2	CIN s8,10 t2			
A	s1,3 t1	t1	s1,2,4-10 t1,2	s1-4,6-10 t1,2	LIM s1-4,6,8,9 t1		
LAG	s1-10 t2	s1,3,9	s2-6,9,10 t1	s1-10 t2	s1-10 t2	A s1-10 t1,2	
CAT	s1-10 t1,2	s1-7,9,10 t1,2	s10	s1-10 t1,2	s1-10 t1,2	s1-10 t1,2	LAG s2,4-7,9,10 t1,2

KEY:

species
PAP = *B. papyracea*
B = *B.* sp. B
PUB = *B. pubescens*
CIN = *B. cincta cincta*
LIM = *B. cincta limbosa*
A = *B.* sp. A
LAG = *B. lagenaria*
CAT = *B. catarrhacta*

variables
s1 = shell length
s2 = shell width
s3 = spire height
s4 = aperture length
s5 = aperture width
s6 = shoulder height
s7 = body weight
s8 =shell weight
s9 = opercular length
s10 = opercular width

t1 = shell thickness 1
t2 = shell thickness 2

The means of a series of ratios were calculated for each species enabling size-independent comparisons of the species. For most of the ratios, there were only small differences between the species (Fig. 4). *B. lagenaria* and *B.* sp. A were very similar to each other in terms of shell width (sw/sl), spire (sp/sl), shoulder height (ht/sl) and aperture length (al/sl), but different from the other six species, being wider with shorter spires, and having a higher shoulder and longer apertures (Fig. 4a-d). *B. lagenaria* and *B.* sp. A also had thicker outer lips (t1/sl) than the other species (Fig. 4e), and *B. c. limbosa* and *B.* sp. A also had a relatively thick deep lip (t2/sl). Opercular length and width (ol/sl, ow/sw) were very similar among the species, although *B. pubescens* and *B. catarrhacta* had relatively small opercula (Fig. 4f). There was more variability between the species for shell and body weights (swt/sl, bwt/sl) (Fig. 4g). *B. c. cincta* and *B. c. limbosa* had the heaviest shells, followed by *B.* sp. A, whilst *B. catarrhacta* and *B. pubescens* had the lightest shells, these being only about a third of the weight of *B. c. cincta*. *B. catarrhacta* and *B. pubescens* also had a lighter body weight than the other species (Fig. 4g).

Although the means of the six qualitative variables are shown in Table 3, frequency histograms of the different states (Fig. 5) for each these variables show some interesting features. The data for the four states of rib strength (RIB1) are shown in Fig. 5a, and of particular note was the result for *B. papyracea*. The bimodal distribution was due to regional differences between the four populations sampled. Approximately half of the snails were collected on the West Coast (Table 1), and had smooth shells, and the other half were collected in the Western Overlap where the shells generally had fairly strong ribs. This bimodality in *B. papyracea* was also reflected in the number of ribs (RIB2 - Fig. 5b) since smooth shells have no ribs. *B. c. cincta*, *B. c. limbosa* and *B. lagenaria* also exhibited clear variations for RIB1, and *B. papyracea* and *B. c. limbosa* for RIB2, again due to the fact that smooth shells also have no ribs. *B.* sp. B was the only species to be classified as having many ribs (Fig. 5b), and although the sample size of this species was relatively small, and rib number can be variable, this character appears to be unique for this species.

The histograms for constriction of the outer lip (Fig. 5c) showed that only *B. lagenaria* and *B. c. cincta* had significant numbers of shells with a marked constriction, whilst only *B. papyracea*

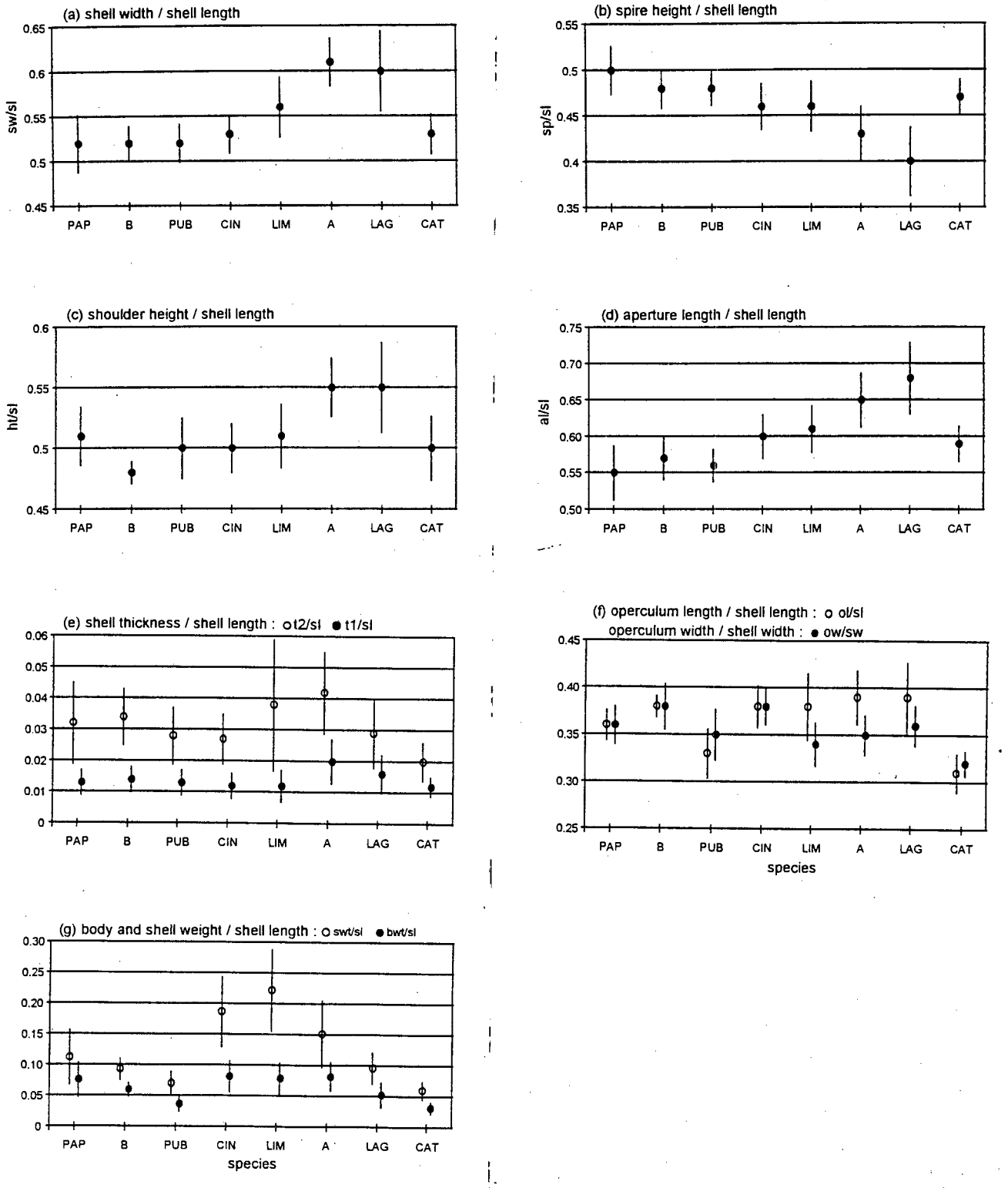


Figure 4. Means (± one standard deviation) of the ratios for each of the species of *Burnupena*.

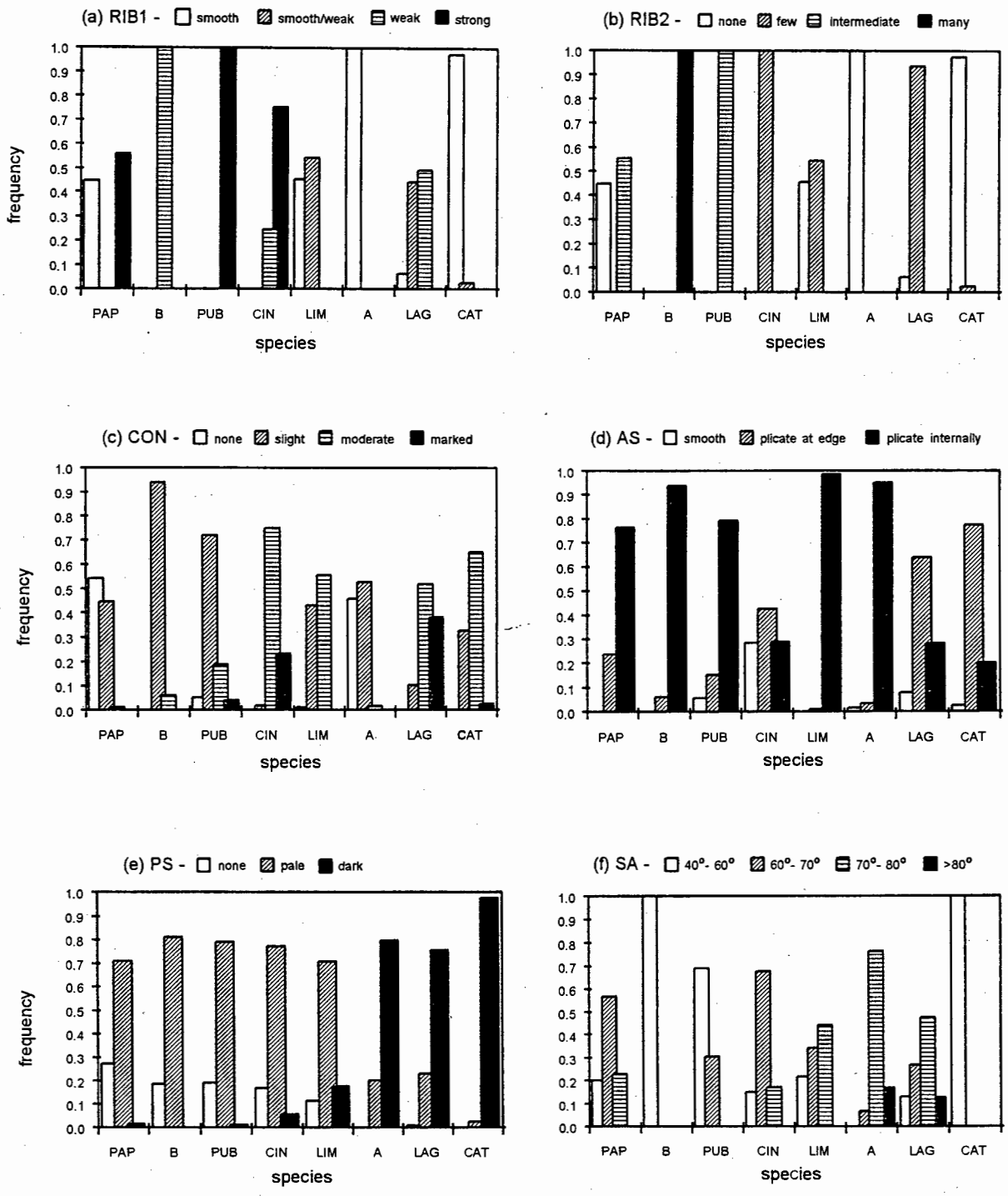


Figure 5. Frequency histograms for the categories of the six qualitative variables for each of the species. (a) rib strength, (b) number of ribs, (c) constriction, (d) aperture sculpture, (e) parietal scar and (f) spire angle.

and *B.* sp. A had reasonable numbers of snails with no constriction. The results for aperture sculpture (Fig. 5d) showed that all species had individuals with internal ridges, but that the most common sculpture for *B. lagenaria, B. catarrhacta* and *B.* c. *cincta* was ridges at the outer lip only. In relation to the parietal scar, the species separated into two groups. *B.* sp. A, *B. lagenaria* and *B. catarrhacta* always had scars, which were generally dark, while the other five species generally had pale scars or lacked scars (Fig. 5e). The angle of the spire was somewhat varied for most of the species, except *B.* sp. B and *B. catarrhacta* which both had acutely pointed spires (Fig. 5f). Only *B. lagenaria* and *B.* sp. A had individuals that had very obtuse spires (spire angle > 80°). For each of these qualitative variables, the species can be grouped into categories, but these grouping are not consistent across variables.

A discriminant analysis using 18 variables was done on all of the individuals, using species as the grouping variable. Two of the 18 variables, shell length (SL) and shell width (SW), were not selected for the classification function as they did not add to the discrimination. The jackknifed classification function correctly identified 94% of the individuals (Table 5a), with all of the individuals belonging to *B. pubescens, B.* sp. A, *B. catarrhacta* and the new species *B.* sp. B, correctly identified. Most of the *B. papyracea* individuals that were misallocated were identified as being more similar to *B. pubescens*, with a couple placed with *B.* sp. A. The incorrectly identified *B.* c. *cincta* individuals were placed into *B.* c. *limbosa*, whilst the misallocated *B.* c. *limbosa* were placed with *B.* sp. A. *B. lagenaria* appeared to be more varied, with misallocated individuals being placed with four of the other species, namely *B.* c. *limbosa, B.* sp. A, *B.* c. *cincta* and *B. catarrhacta*.

Figure 6a shows a plot of the first and second canonical variables for each of the species, together with the species' means. It is clear that there is a large amount of overlap between most of the species as indicated by the overlap of the convex hulls. Most of the separation between the species was along the axis of the first canonical variable, which accounted for about 50% of the total variance. The second canonical variable accounted for about 24% of the total variability, and there was less separation of the species along this axis (Fig. 6a). The standardized coefficients of the

Table 5. Discriminant and canonical variate analysis for eight species of *Burnupena*. Jackknifed classification (a) and standardized coefficients (b) using all 18 variables. Jackknifed classification (c) and standardized coefficients (d) using only the quantitative variables. For species abbreviations refer to Table 4.

(a) Jackknifed classification.

species	%correct	no. of cases classified into species								
		PAP	B	PUB	CIN	LIM	A	LAG	CAT	n
PAP	86.7	124	-	**17**	-	-	**2**	-	-	143
B	100.0	-	15	-	-	-	-	-	-	15
PUB	100.0	-	-	50	-	-	-	-	-	50
CIN	98.4	-	-	-	180	**3**	-	-	-	183
LIM	91.4	-	-	-	-	53	**5**	-	-	58
A	100.0	-	-	-	-	-	59	-	-	59
LAG	91.3	-	-	-	**3**	**5**	**5**	179	**4**	196
CAT	100.0	-	-	-	-	-	-	-	39	39
total	94.1	124	15	67	183	61	71	179	43	743

The values in bold indicate the numbers of animals placed into the wrong group.

(b) Standardized coefficients for canonical variables.

	Canonical variable 1	Canonical variable 2
variable		
spire	**-1.50**	**0.77**
aperture length	0.73	**0.73**
aperture width	0.14	-0.60
shoulder height	-0.50	0.56
shell thickness 1	-0.04	-0.13
shell thickness 2	-0.55	0.37
body weight	-0.73	**-0.99**
shell weight	**1.79**	**-1.10**
opercular length	0.40	0.44
opercular width	0.17	0.61
rib strength	**1.81**	**0.79**
no. ribs	**-1.45**	-0.50
constriction	0.37	-0.48
aperture sculpture	-0.15	0.02
parietal scar	0.14	-0.42
spire angle	-0.33	-0.10
cumulative % of total variance	50.0	74.3

The values in bold indicate the variables that contribute most to the separation of the groups.

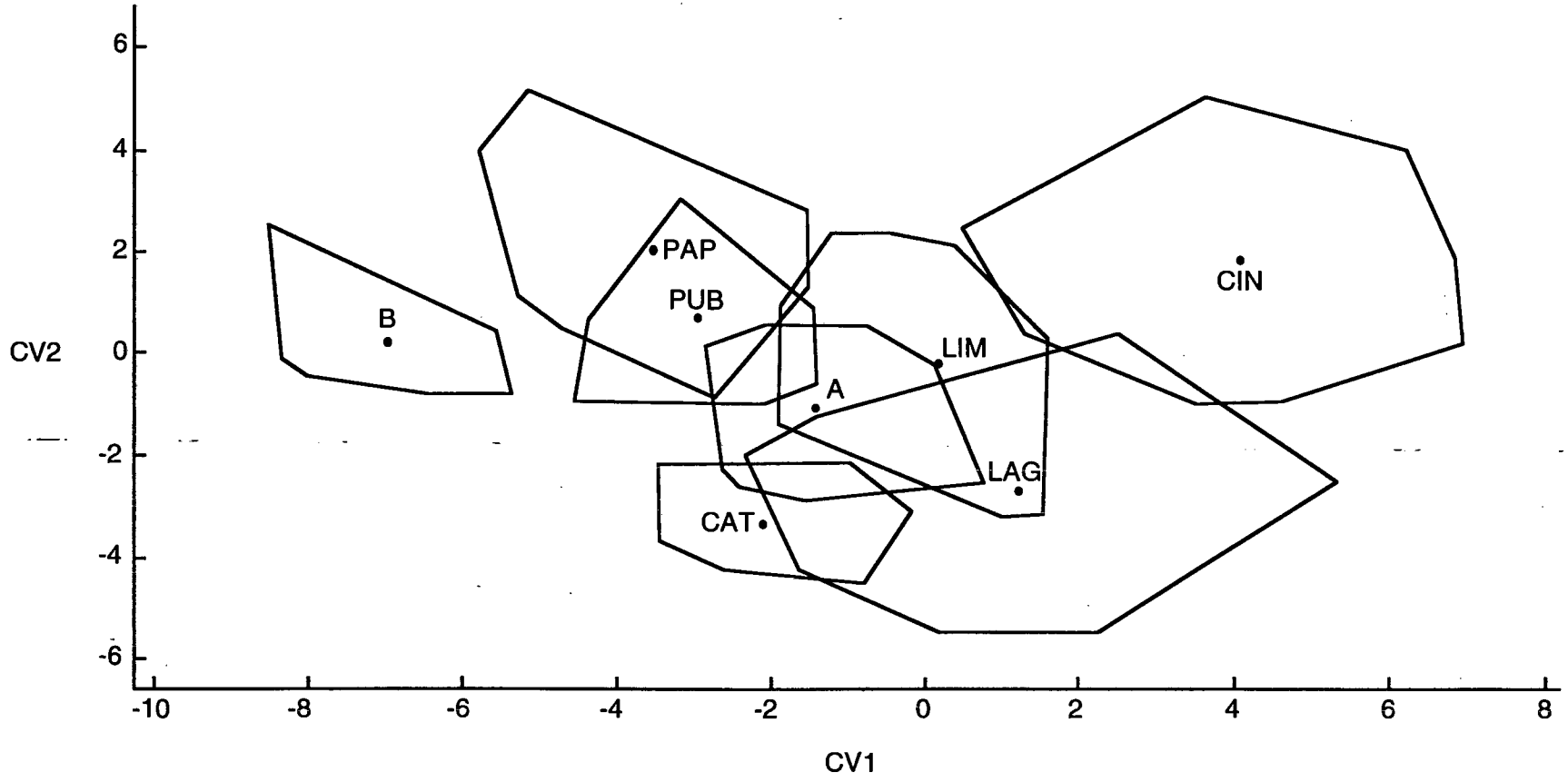


Figure 6a. Plot of the first two canonical varibles (CV1 and CV2) for the first discriminant analysis with all species and all 18 variables. Centroids for each species are as follows: PAP = *B. papyracea*, CIN = *B. cincta cincta*, PUB = *B. pubescens*, LAG = *B. lagenaria*, LIM = *B. cincta limbosa*, CAT = *B. catarrhacta*, A = *B.* sp. A and B = *B.* sp. B. Outlines are convex hulls surrounding 100% of the points for that species.

canonical variables are given in Table 5b. Their magnitudes can be used to indicate the relative contribution of the variables to the canonical variable. The first canonical variable had large positive standardized coefficients for rib strength and shell weight, and large negative coefficients for spire height and number of ribs, indicating that the separation between the species along this axis is achieved mainly by a contrast of these variables. The interpretation of the second canonical variable is less obvious. Body and shell weights had the largest negative coefficients, and spire, aperture length and rib strength had the largest positive coefficients (Table 5b). Although the other variables (of both canonical variables) have smaller coefficients, they are not without effect, and do play a role in the final placement of the individuals.

Considering both axes simultaneously, the species can be separated into four groups with varying amounts of overlap between them (Fig. 6a). The new species, *B.* sp. B, forms a group by itself, and is the only group which was clearly distinct. *B.* c. *cincta* also is in a group virtually on its own. The third group comprises *B. papyracea* and *B. pubescens*, and the fourth group consists of *B. lagenaria*, *B.* c. *limbosa*, *B.* sp. A and *B. catarrhacta*.

The discriminant and canonical analyses were repeated using only the 12 quantitative variables (Table 5c,d and Fig. 6b). Two of the 12 variables, shell width and aperture length, were omitted by the discriminant analysis as they contributed nothing further to the discrimination. The results show that for all species except *B. catarrhacta*, a lower percentage of snails were allocated to the correct species than when the qualitative variables were included (Table 5c). The jackknifed classification function correctly identified about 82% of the individuals as opposed the 94% using all variables (Table 5a). A large number of *B. papyracea* individuals were allocated to *B. pubescens*, as occurred using all variables. Also of note was the large number of *B.* sp. A snails allocated to *B. lagenaria* and vice versa, as well as a fair number of *B.* c. *cincta* allocated to *B.* c. *limbosa* and vice versa.

The variables that contributed most to the separation of the species along the first canonical axis were shell length and shell weight, which had large negative coefficients, and spire height,

Table 5 continued. Discriminant and canonical variate analysis for eight species of *Burnupena*.

(c) Jackknifed classification.

species	%correct	no. of cases classified into species								
		PAP	B	PUB	CIN	LIM	A	LAG	CAT	n
PAP	75.5	109	**8**	**19**	**2**	-	**4**	**2**	-	144
B	93.3	**1**	14	-	-	-	-	-	-	15
PUB	88.0	-	**3**	44	-	-	-	-	**3**	50
CIN	92.3	-	**1**	-	169	**11**	**1**	**1**	-	183
LIM	72.9	**1**	-	**1**	**8**	43	**4**	**2**	-	59
A	76.3	**1**	-	-	**1**	-	45	**11**	**1**	59
LAG	76.5	-	-	**3**	**8**	**4**	**27**	156	**6**	204
CAT	100.0	-	-	-	-	-	-	-	39	39
total	82.2	112	26	67	188	58	81	172	49	753

The values in bold indicate the numbers of animals placed into the wrong group.

(d) Standardized coefficients for canonical variables.

	Canonical variable 1	Canonical variable 2
variable		
shell length	**-2.43**	-0.19
spire	**2.40**	**1.50**
aperture width	1.02	-0.82
shoulder height	1.35	0.84
shell thickness 1	0.30	-0.35
shell thickness 2	0.52	0.54
body weight	0.66	**-1.04**
shell weight	**-2.25**	**-1.36**
opercular length	-0.56	0.79
opercular width	-0.85	0.80
cumulative % of total variance	45.1	79.8

The values in bold indicate the variables that contribute most to the separation of the groups.

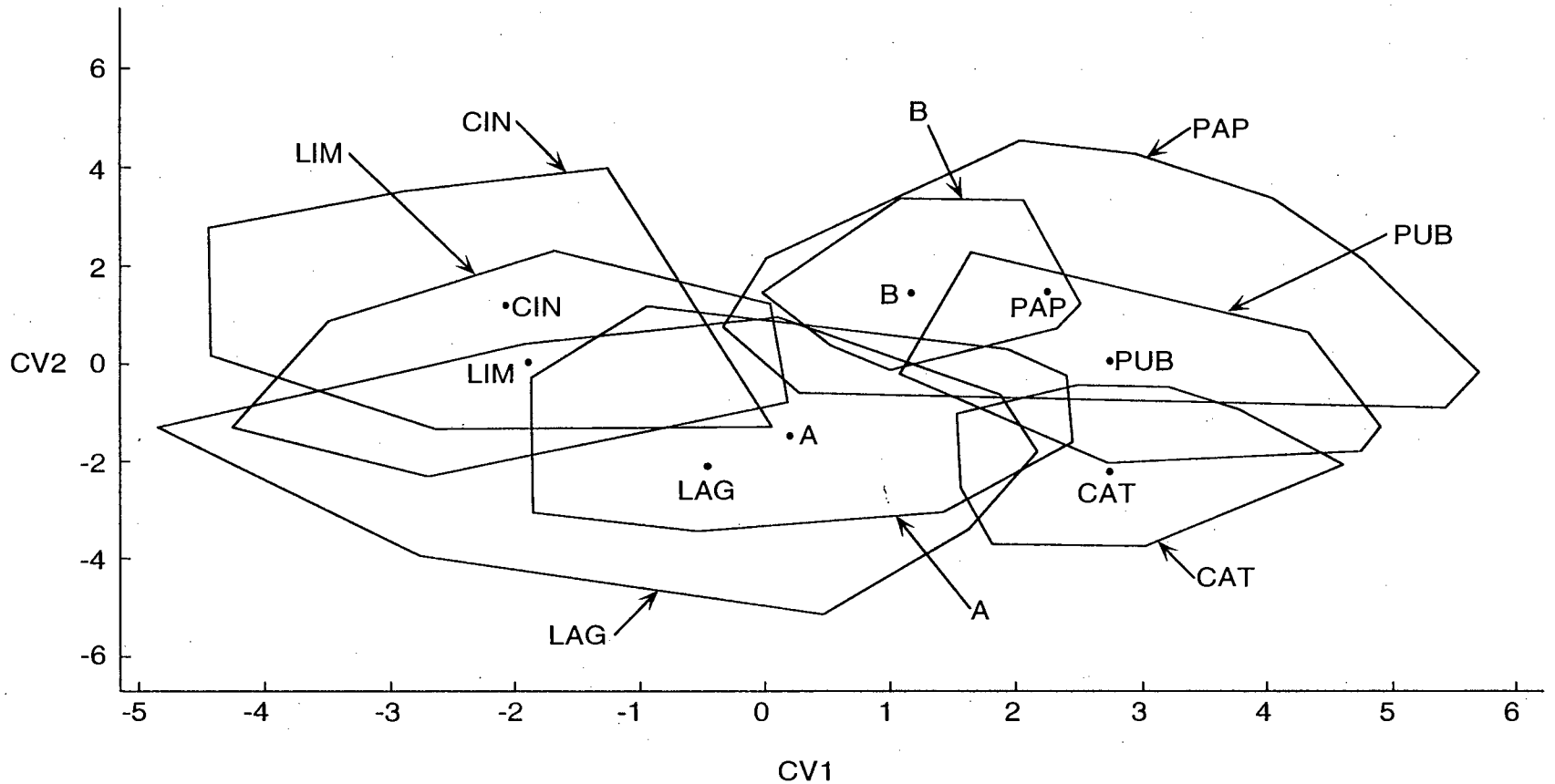


Figure 6b. Plot of the first two canonical variables (CV1 and CV2) for the second discriminant analysis for all species and using the 12 quantitative variables only. Centroids for the species are as in Figure 6a. In order to clarify the species, the outlines are labelled.

which was large and positive. Along the second axis, spire was also large and positive, whilst body and shell weights had large negative coefficients (Table 5d). The plot of these two canonical variables is shown in Fig. 6b. As expected, the degree of overlap between the species was much greater than was found using all of the variables. *B.* sp. B now completely overlapped *B. papyracea*. It's earlier separation was mainly due to the distinct number of ribs of this species (Fig. 5b). Without this variable in the analysis, *B.* sp. B was not distinguishable from *B. papyracea*. *B.* c. *cincta* now showed almost complete overlap with *B.* c. *limbosa* (and partial overlap with both *B. lagenaria* and *B.* sp. A).

The principal component analysis reduced the original 12 quantitative variables into a small number of components, the first of which accounted for about 83% of the total variation, the second for about 10%, and the third component for approximately 3% (Table 6). Thus, the first three components accounted for about 96% of the total variance. The elements of the first eigenvector are positive, approximately of equal size and most are very close to the predicted value of (number of characters)$^{-1/2}$ which equals 0.29. The values for T1 and T2 are not as close as the other variables, indicating the lower correlation between these two variables and the others. My interpretation is that the first principal component mainly represents variation due to size. This can more clearly be seen from the position of the species along this axis in the plot of principal components (Fig. 7a-b). Those species with a smaller shell size (length and width) have lower scores for the first component. There was no separation of any of the species along the first axis, although the overlap between a few of the species, mostly involving *B. catarrhacta*, was not very large .

As expected from their lower correlations with the other variables, most of the variation of the second component (Table 6) was due to the thickness of the shell at the aperture (T1 and T2). There was substantial overlap between all of the species along this axis (Fig. 7a). The height of the spire contributed most to the variation in the third component. After T1 and T2, this variable had the lowest correlations with the other variables, as previously noted. There was also no separation between the species along the axis of the third component (Fig. 7b), although there was less overlap between some of the species than was seen along the axis of the second component. *B. papyracea*,

Table 6. Principal component coefficients, eigenvalues and cummulative percentages of the total variance for all species of *Burnupena*.

Component:	1	2	3
Eigenvalue:	10.00	1.15	0.39
Cumulative %:	83.3	92.9	96.2
Eigenvectors:			
variable			
shell length	0.303	-0.172	0.316
shell width	0.313	-0.049	-0.155
spire height	0.270	-0.202	**0.745**
aperture length	0.305	-0.116	-0.296
aperture width	0.306	-0.006	-0.264
shoulder height	0.311	-0.090	0.012
shell thickness 1	0.176	**0.735**	-0.046
shell thickness 2	0.220	**0.590**	0.291
body weight	0.307	-0.089	-0.034
shell weight	0.307	0.024	-0.042
operculum length	0.306	-0.091	-0.140
operculum width	0.305	-0.032	-0.234

Values in bold indicate the variables that contribute the most to the variation in each component.

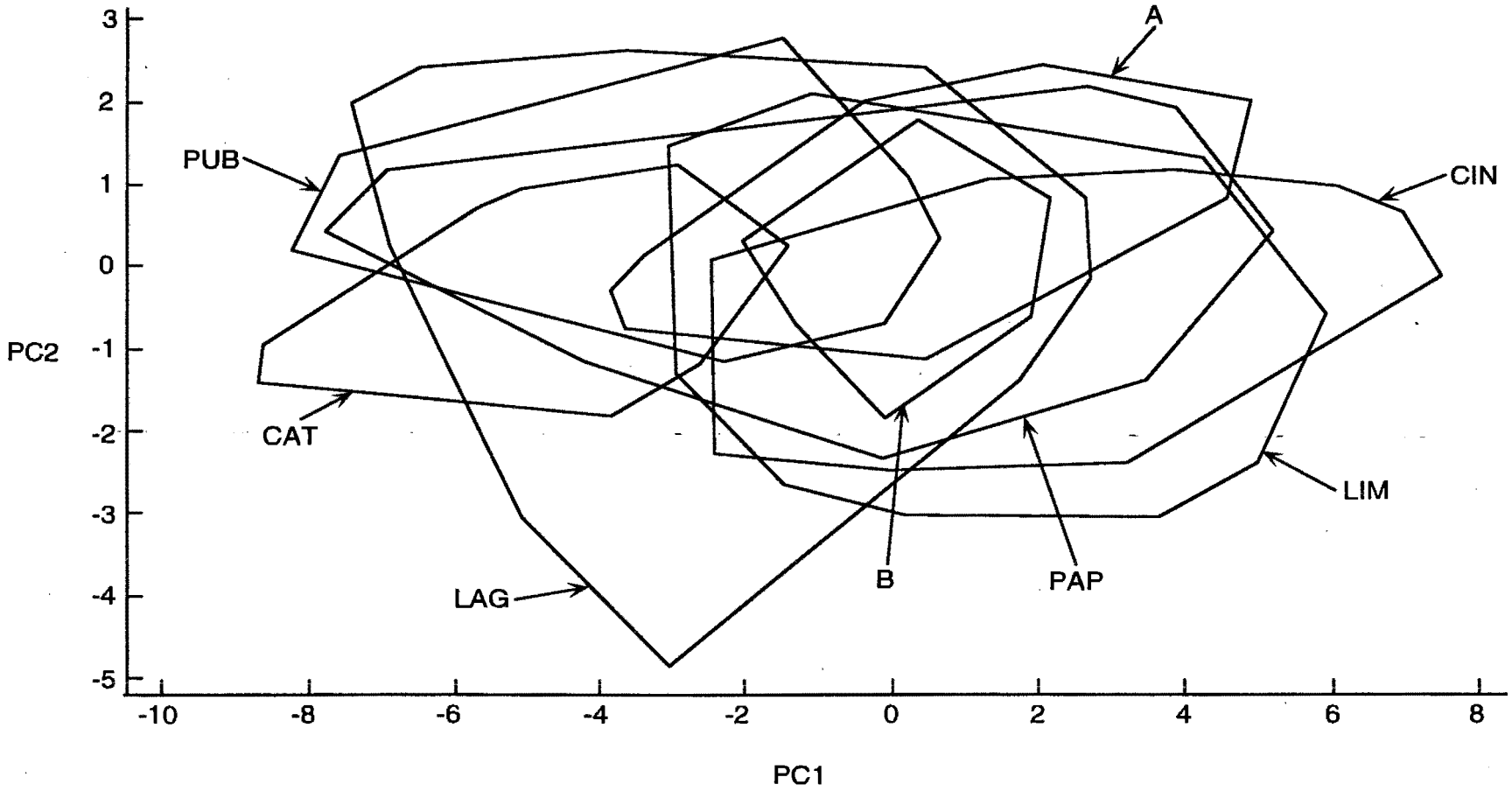


Figure 7a. Plot of the principal component scores of the first two components (PC1 and PC2) for all of the species. Only the group enclosing lines are displayed. Species labels are as in Figure 6a.

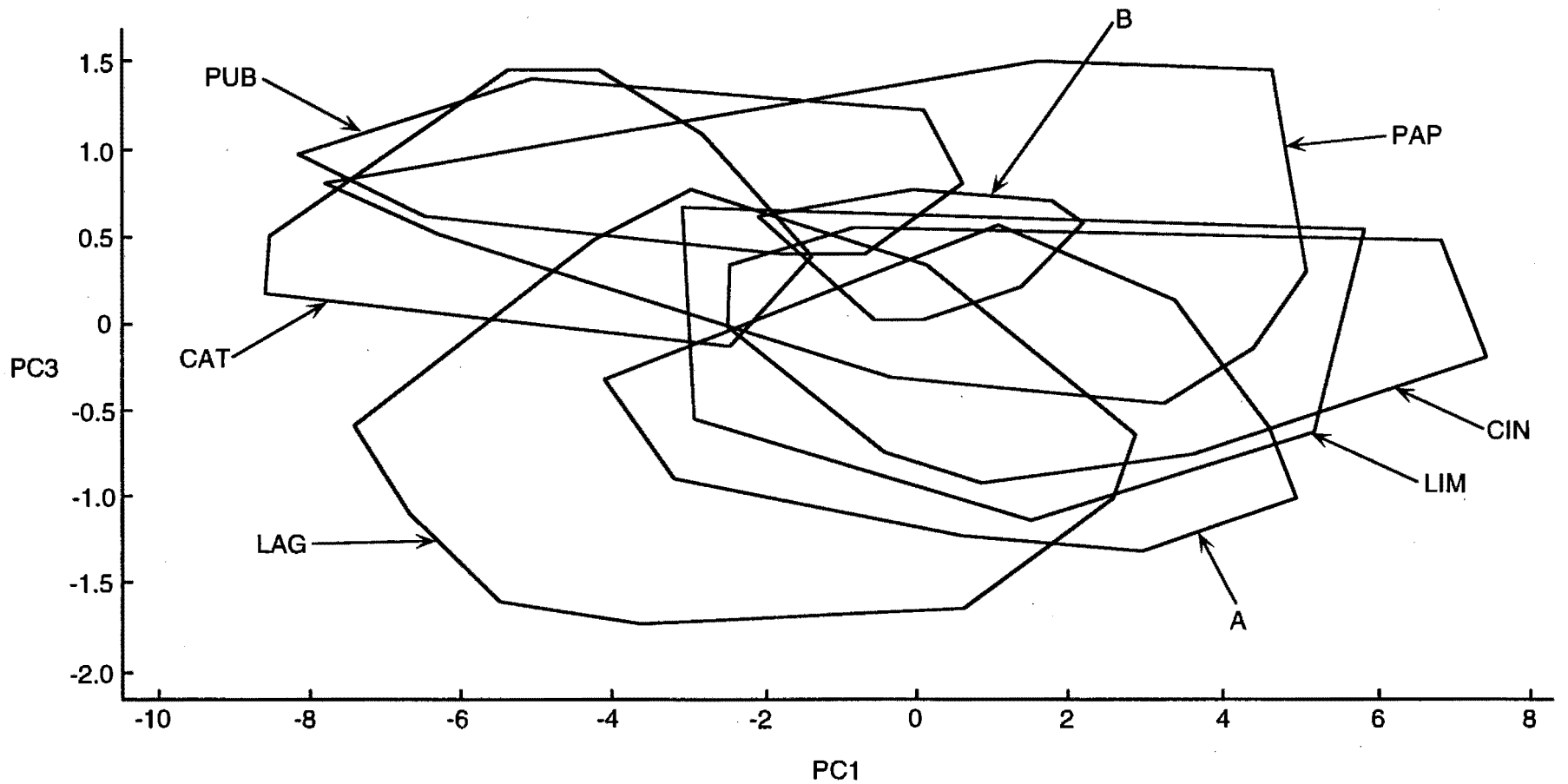
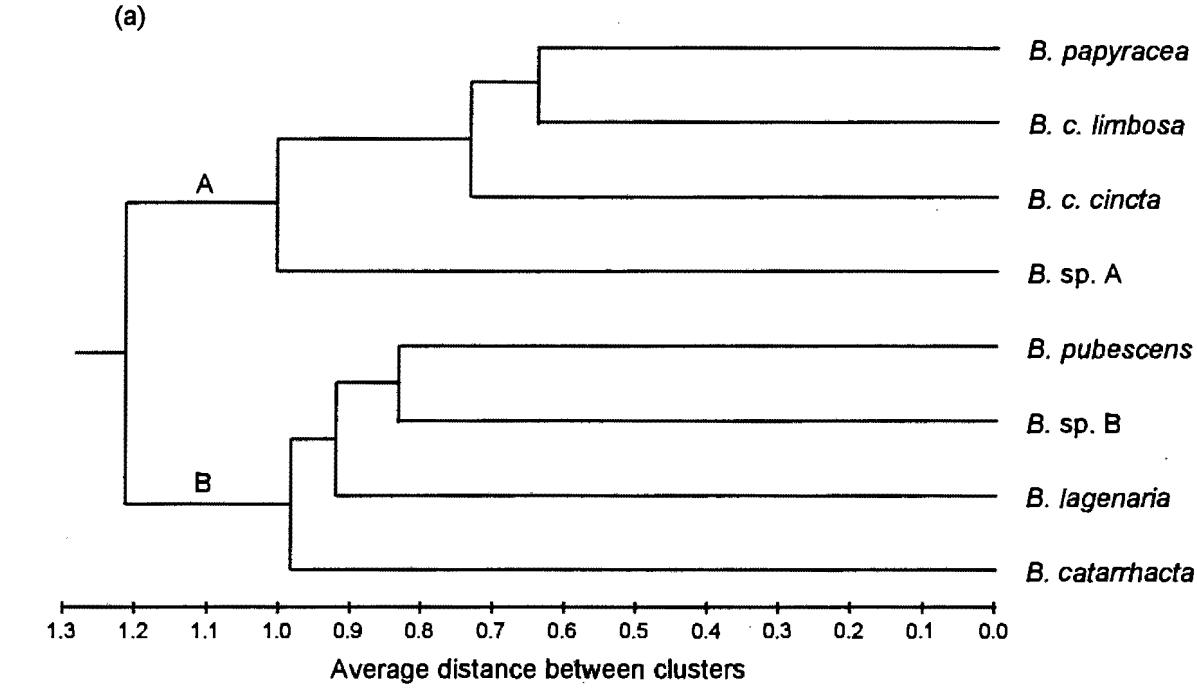


Figure 7b. Plot of the principal component scores for the first and third components (PC1 and PC3) for all of the species. Only group enclosing lines are shown. Species abbreviations are as in Figure 6a.

B. pubescens, *B. catarrhacta* and *B.* sp. B were plotted above the other four species, reflecting the relatively higher spires possessed by these species, as shown in Fig. 4b. Although the second component accounted for about 10% of the total variance, and the third for only about 3%, the greater overlap between the species along the second axis implies that a large part of the variability of T1 and T2 is found within species, rather than between species. It can be seen from Table 3 and Fig. 4e that the standard deviations of these two variables were relatively high in all of the species.

Whilst the first principal component appears to represent variation due to size, none of the separation along the axes of the canonical variables (Fig. 6a-b) seemed solely attributable to size, as determined by mean shell length (Fig. 3). The discriminant analysis uses the known structure of the data, that is, the different species, and takes within-group variation into consideration. It therefore minimizes within-species variation and emphasizes the differences between the species. Principal component analysis does not use group information, and within-species variation is not distinguished from between-species variation. The differences in approach can be seen from the better separation between the species using the canonical variables (Fig. 6a-b). The results from the principal components analysis show, however, that the amount of variability within species is high relative to the variability of the genus as a whole.

The cluster analysis, using the means for each species, was run twice. In the first analysis all 18 variables were used, and in the second, the six qualitative variables were excluded in order to assess their effect. The resulting dendrograms are shown in Fig. 8. A comparison of the two dendrograms reveals a number of differences, not only in the groups that were formed, but also in the average distance between the clusters. In the 18-variable run, two main clusters of four species each were formed (Fig. 8a). *B. papyracea*, *B. c. cincta*, *B. c. limbosa* and *B.* sp. A (cluster A) were separated from *B. pubescens*, *B.* sp. B, *B. lagenaria* and *B. catarrhacta* (cluster B). These groupings partially reflected the overall size of the species. The distances at which the species/clusters were joined were relatively large, with the minimum distance between any two species (0.64 between *B. papyracea* and *B. c. limbosa*) approximately the same as the maximum distance between any two groups (0.62 between *B. papyracea* and cluster B). In the run excluding the qualitative variables



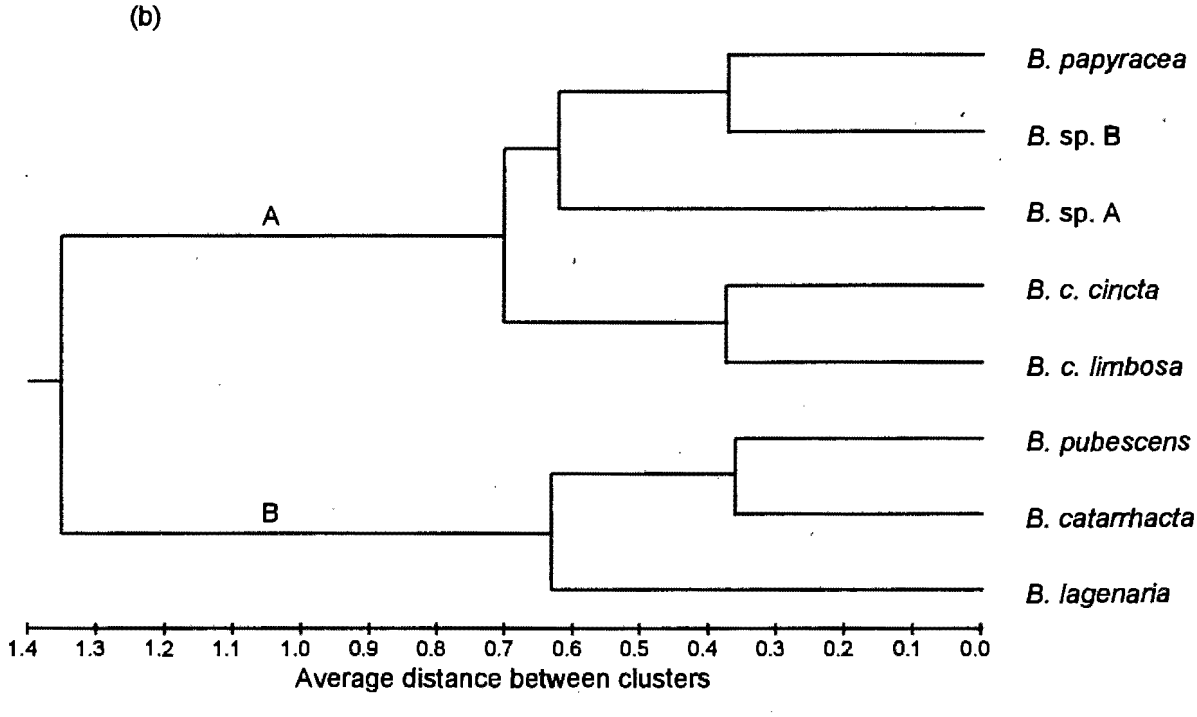


Figure 8. Dendrograms showing the affinities of the species of *Burnupena* based on (a) all 18 variables and (b) using only the 12 quantitative variables.

(Fig. 8b), two main clusters were again produced with the same species in each, except *B.* sp. B which was grouped with the species in cluster A. The clusters formed here clearly reflected differences in overall size. As the principal components analysis revealed, over 80% of the total variation in the quantitative variables was due to differences in size. The species were also more similar as shown by the much smaller distances at which the species/clusters were joined. This confirms the comment made above that grouping of species based on the qualitative variables were not consistent across variables.

In summary, there were significant differences between most of the pairs of species for at least one of the quantitative size and thickness variables, although in most cases the relative importance of the variables differed. There were only small differences in shell shape between the species, although they differed more in their relative shell and body weights. In terms of the qualitative variables, all species exhibited a wide range of states, except for the two rib variables, which proved to be diagnostic in some instances.

In the discriminant analysis, 94% of the individuals could be allocated to the correct species. Despite this, there was a large amount of overlap between most of the species when the first two canonical variables were plotted, although a number of groups could be discerned. The qualitative variables, especially the two rib variables, contributed substantially to the separation between the species. Since it failed to separate the species, the principal components analysis revealed that the within-species variability is high, a factor of considerable importance that is explored in more detail for each of the species in the following section.

VARIATION WITHIN SPECIES

Size differences between sexes

B. papyracea

In all four populations of *B. papyracea*, the mean size of the males was smaller than that of the females, for each the 12 variables examined (Table 7). For Bakoven and A-Frame the differences were significant for all of the variables (t-tests: $p<0.001$ and $p<0.05$) except for the two measures of shell thickness. For Blouberg and Castle Rock, although the males were always smaller than the females, these differences were only significant ($p<0.05$) for five and four of the 12 variables respectively. For both these populations, shell length was not significantly different between the sexes.

The mean size of the snails from Castle Rock, both male and female, was much smaller than the other three populations, and this population also had a greater within-sample variance. It is not certain whether the greater variability and overall smaller size of the CR population was an artefact of the sampling procedure, or whether it reflects the real nature of this population. Castle Rock and A-Frame are only about 4km apart and would be expected to have similar environments.

B. cincta cincta and *B. cincta limbosa*

Five populations of *B. cincta cincta* and three of *B. cincta limbosa* were considered in these analyses. As with the populations of *B. papyracea*, the mean size of the males was smaller than that of the females, for each the 12 variables tested, and for most of the populations (Table 8). Two of the populations, Blouberg and Port Elizabeth, showed no significant differences between the males and females for any of the variables. There was also no significant difference between sexes at Kommetjie except for opercular length. However, of the 32 individuals sampled, only three were

Table 7. Means and standard deviations for populations of *B. papyracea*. The values shown in bold indicate significant differences between the sexes (m=males, f=females).

	Blouberg (BB)		Bakoven (BO)		Castle Rock (CR)		A-Frame (AF)	
	(m)	(f)	(m)	(f)	(m)	(f)	(m)	(f)
shell length	43.21	45.78	**42.66**	**46.64**	30.15	33.64	**41.29**	**47.88**
(sl)	5.25	5.46	3.66	3.36	4.69	7.21	6.93	5.70
shell width	**23.16**	**24.97**	**23.33**	**25.31**	**15.67**	**17.66**	**20.36**	**23.34**
(sw)	2.60	2.62	1.49	1.53	2.10	3.39	2.87	2.18
spire height	20.85	22.48	**20.72**	**23.02**	14.72	16.49	**21.56**	**25.40**
(sp)	2.76	2.95	2.19	2.04	2.66	4.07	4.39	3.83
aperture	24.85	26.37	**24.92**	**26.78**	**16.92**	**18.80**	**21.36**	**24.29**
length (al)	2.98	2.78	1.81	1.66	2.18	3.14	2.84	2.12
aperture	11.70	12.62	**11.78**	**12.99**	**7.40**	**8.43**	**9.95**	**11.64**
width (aw)	1.66	1.60	0.78	0.91	1.11	1.70	1.51	1.23
shoulder	**21.53**	**23.36**	**22.19**	**24.39**	15.48	17.24	**21.99**	**24.37**
height (ht)	2.79	2.89	1.57	1.65	2.21	3.38	1.03	1.21
thickness 1	0.58	0.51	0.72	0.76	0.37	0.37	0.54	0.54
(t1)	0.25	0.21	0.16	0.26	0.08	0.11	0.14	0.16
thickness 2	1.24	1.19	2.02	2.01	0.82	0.84	1.23	1.37
(t2)	0.56	0.66	0.68	0.65	0.22	0.26	0.43	0.50
body weight	**3.67**	**4.76**	**3.58**	**4.67**	**1.11**	**1.72**	**2.89**	**4.30**
(bwt)	1.20	1.80	0.97	0.93	0.49	1.06	1.18	1.22
shell weight	6.07	6.44	**5.81**	**7.27**	1.70	2.40	**4.14**	**5.83**
(swt)	2.56	2.65	1.60	1.58	0.86	1.57	1.47	1.92
opercular	16.00	16.65	**15.56**	**17.05**	10.53	11.59	**15.79**	**17.77**
length (ol)	2.13	1.82	1.37	1.01	1.83	2.60	1.00	1.07
opercular	**8.31**	**9.00**	**8.52**	**9.19**	**5.73**	**6.51**	**8.18**	**9.05**
width (ow)	1.02	0.89	0.81	0.55	0.86	1.29	0.35	0.59
n =	14	17	22	28	20	22	24 ht=13 ol=13 ow=13	34 ht=17 ol=,17 ow=17

Table 8. Means and standard deviations for populations of *B. cincta*. The values shown in bold indicate significant differences between the sexes (m=males, f=females).

	B. cincta limbosa					*B. cincta cincta*								
	Blouberg (BB)	Oudekraal (OK) (m)	(f)	Kommetjie (KM) (m)	(f)	Castle Rock (CR) (m)	(f)	A-Frame (AF) (m)	(f)	Dalebrook (DK) (m)	(f)	Mossel Bay (MB) (m)	(f)	Port Elizabeth (PE)
shell length	44.93	**38.52**	**48.29**	44.88	47.94	**46.67**	**53.92**	**44.98**	**53.41**	**41.67**	**46.13**	**42.27**	**47.61**	43.66
(sl)	3.18	4.72	4.52	2.84	7.13	5.69	7.20	6.43	7.51	4.66	4.34	6.87	5.76	4.65
shell width	26.33	**21.79**	**26.96**	23.38	25.32	**24.59**	**28.23**	**24.04**	**27.85**	**22.50**	**24.81**	**22.49**	**24.95**	23.36
(sw)	2.16	2.68	2.44	1.08	2.97	2.97	3.16	3.19	3.63	2.44	1.78	3.97	2.80	2.48
spire height	20.12	**16.94**	**22.14**	20.51	22.61	**21.91**	**25.73**	**21.25**	**25.71**	**19.25**	**21.69**	**18.06**	**21.27**	19.21
(sp)	2.22	2.40	2.38	1.35	4.34	2.89	3.94	3.42	4.11	2.20	2.65	3.15	3.28	2.49
aperture	27.57	**24.08**	**29.12**	26.99	28.28	**27.55**	**31.07**	**26.36**	**30.50**	**24.80**	**26.91**	**26.46**	**29.19**	26.96
length (al)	1.80	2.77	2.48	1.24	3.21	3.28	3.81	3.10	3.54	2.93	2.03	3.85	2.86	2.74
aperture	13.06	**10.22**	**12.83**	11.29	12.16	**11.78**	**13.57**	**11.20**	**13.35**	**10.88**	**12.17**	**10.72**	**12.04**	10.84
width (aw)	1.08	1.28	1.34	0.56	1.68	1.76	1.71	1.64	2.06	1.38	1.12	1.82	1.51	1.28
shoulder	23.50	**18.97**	**22.92**	22.55	24.14	**22.84**	**26.72**	**21.39**	**25.62**	**20.44**	**22.56**	**21.36**	**24.24**	22.30
height (ht)	2.06	2.77	1.71	0.81	3.25	2.85	3.51	3.73	3.49	2.28	1.86	2.96	2.56	2.21
thickness 1	0.76	0.49	0.68	0.36	0.40	0.64	0.64	0.50	0.56	0.44	0.48	0.64	0.65	0.55
(t1)	0.18	0.19	0.36	0.09	0.17	0.27	0.22	0.23	0.22	0.12	0.15	0.27	0.23	0.23
thickness 2	1.60	**2.16**	**2.98**	0.83	1.09	1.65	1.70	1.17	1.55	1.08	1.18	1.21	1.32	0.85
(t2)	0.45	0.76	1.05	0.25	0.67	0.37	0.51	0.38	0.71	0.33	0.42	0.34	0.31	0.34
body weight	4.38	**1.93**	**4.18**	3.00	4.10	3.51	4.92	**3.57**	**5.87**	**3.23**	**4.18**	**2.74**	**3.61**	3.20
(bwt)	1.09	0.72	1.33	0.66	1.73	1.32	1.64	1.44	2.33	1.23	1.05	1.33	1.15	0.98
shell weight	9.25	**7.78**	**14.20**	8.63	10.93	**8.79**	**13.80**	**8.55**	**12.18**	**6.04**	**7.89**	8.74	9.78	7.17
(swt)	2.91	3.13	5.04	1.31	4.32	3.00	5.22	3.65	4.74	1.99	2.32	3.09	3.71	2.22
opercular	19.05	**14.15**	**17.83**	**14.85**	**17.10**	**18.18**	**21.29**	**16.96**	**20.16**	**15.68**	**17.17**	**15.73**	**17.98**	17.00
length (ol)	1.59	1.70	1.65	0.60	2.47	2.46	3.13	2.91	2.83	2.09	1.55	2.62	2.37	1.94
opercular	9.88	**7.14**	**9.07**	7.62	8.40	**9.32**	**10.69**	**8.57**	**10.34**	**8.73**	**9.69**	**8.51**	**9.58**	8.95
width (ow)	0.74	0.93	0.98	0.46	1.08	1.23	1.37	1.38	1.47	0.98	0.75	1.25	1.03	1.10
n =	18	16 ht=7	13 ht=3	3	29	7	14	22 ht=9 ol=9 ow=9	35 ht=31 ol=31 ow=31	21	41	20 swt=15	25	22

male, so this result is questionable. For the remaining five populations, Oudekraal, Castle Rock, A-Frame, Dalebrook and Mossel Bay, there were significant differences ($0.05 < p > 0.001$) between the sexes for most of the variables except for shell thickness (T1 and T2).

B. pubescens

As noted in the introduction, species identifications were confirmed by the allozyme electrophoretic analysis. It was found that three individuals from CR which were initially classified as *B. papyracea* based on their shell morphology, were in fact *B. pubescens*. Subsequently, the initial species assignment was modified, and these individuals have been incorporated within *B. pubescens* in the analyses given below.

Four populations of this species were analysed, although two of them, Rooiels and Port Elizabeth, had only a few individuals (seven and five respectively) and were omitted from some of the analyses. In three of the populations (Castle Rock, RE and PE), the mean size of the males was smaller than that of the females (Table 9), although the differences were marginal in the PE population and are not presented. For the CR population, these differences were significant ($p < 0.05$) for seven of the 12 variables.

B. lagenaria

The differences in size between the sexes within the *B. lagenaria* populations were not as distinct as in the previous species: there were significant differences between the sexes in only four of the eight populations (Table 10). In three of these (Kommetjie, Dalebrook and Durban), females had significantly greater shell widths ($p < 0.05$). In the DN population, there was also a significant difference between the sexes for shoulder height and shell thickness 1, with the females being larger (HT) but thinner (T1). At Mossel Bay, the females were significantly smaller than the males

Table 9. Means and standard deviations for populations of *B. pubescens*. The values in bold indicate significant differences between the sexes (m=males, f=females).

	Castle Rock (CR)		A-Frame	Rooiels (RE)		Port Eliza-
	(m)	(f)	(AF)	(m)	(f)	beth (PE)
shell length	**26.97**	**30.05**	34.18	29.76	33.57	26.47
(sl)	3.92	2.89	3.57	0.75	3.46	0.31
shell width	**14.23**	**15.77**	17.70	**15.09**	**17.69**	14.02
(sw)	1.99	1.69	1.76	0.46	1.24	0.86
spire height	**12.82**	**14.62**	16.76	13.63	16.08	12.32
(sp)	1.91	1.40	1.96	0.42	1.71	0.41
aperture	**15.60**	**17.00**	18.94	16.42	18.34	15.31
length (al)	2.11	1.66	1.78	1.37	1.68	0.77
aperture	**6.60**	**7.30**	8.43	7.15	8.14	6.43
width (aw)	1.04	0.93	1.01	0.42	0.68	0.38
shoulder	**13.71**	**15.55**	17.75	15.02	15.84	13.59
height (ht)	2.05	1.62	1.87	1.00	1.43	0.71
thickness 1	0.37	0.42	0.44	-	0.31	0.24
(t1)	0.11	0.22	0.09	-	0.11	0.04
thickness 2	0.91	0.92	0.96	-	0.63	0.42
(t2)	0.25	0.37	0.28	-	0.26	0.09
body weight	**0.74**	**1.04**	1.55	-	-	-
(bwt)	0.33	0.37	0.52	-	-	-
shell weight	1.61	1.96	2.76	-	-	-
(swt)	0.69	0.63	0.87	-	-	-
opercular	9.28	9.99	11.28	-	-	-
length (ol)	1.17	1.15	1.14	-	-	-
opercular	5.40	5.57	6.07	-	-	-
width (ow)	0.84	0.62	0.48	-	-	-
n =	14	19	30 ol=20 ow=20 ht=20	2	5	5

Table 10. Means and standard deviations for populations of *B. lagenaria.* The values in bold indicate significant differences between sexes (m=males, f=females).

	Groen River (GR)	Blouberg (BB)	Kommetjie (KM) (m)	(f)	Dalebrook (DK) (m)	(f)	Sparks Bay (SB)	Hermanus (HM)	Mossel Bay (MB) (m)	(f)	Durban (DN) (m)	(f)
shell length	33.06	31.65	32.45	34.12	30.90	31.86	37.49	32.95	**32.53**	**30.15**	22.32	23.39
(sl)	2.79	3.13	3.72	3.20	1.98	2.01	4.46	3.82	2.07	2.16	1.65	1.23
shell width	20.99	19.88	**17.54**	**19.01**	**18.07**	**18.90**	22.25	19.43	**18.06**	**17.32**	**14.79**	**15.73**
(sw)	1.95	2.11	1.95	1.74	1.05	1.16	2.80	2.03	1.03	1.03	1.17	0.75
spire height	12.26	11.82	13.63	14.61	12.39	13.07	15.64	13.22	13.69	13.00	7.66	7.88
(sp)	1.51	1.39	1.74	2.02	0.97	1.23	1.86	2.13	1.08	1.45	0.78	0.78
aperture	23.39	22.36	20.55	21.57	20.62	20.94	24.33	22.15	**20.77**	**19.23**	16.97	17.82
length (al)	1.89	2.21	2.25	1.48	1.23	1.03	2.69	2.20	1.17	1.21	1.33	0.93
aperture	10.92	10.01	8.86	9.50	8.91	9.24	10.97	9.68	**8.74**	**8.24**	7.60	7.98
width (aw)	1.04	1.19	1.00	0.94	0.70	0.74	1.67	1.12	0.62	0.63	0.62	0.57
shoulder	18.63	17.89	17.00	18.03	16.54	17.13	19.43	17.59	16.97	16.28	**13.85**	**14.58**
height (ht)	1.67	1.69	1.81	1.41	0.96	0.86	2.31	1.94	1.08	1.16	1.02	0.67
thickness 1	0.61	0.56	0.28	0.38	0.42	0.46	0.43	0.58	0.57	0.56	**0.56**	**0.45**
(t1)	0.21	0.21	0.16	0.21	0.11	0.15	0.19	0.14	0.18	0.17	0.11	0.11
thickness 2	1.08	1.07	0.46	0.62	0.81	0.88	0.76	1.06	1.00	0.97	0.99	0.90
(t2)	0.39	0.33	0.24	0.33	0.14	0.26	0.32	0.25	0.19	0.17	0.20	0.15
body weight	2.72	1.87	1.55	1.70	1.52	1.75	2.63	1.82	1.19	1.12	0.61	0.71
(bwt)	0.67	0.52	0.51	0.38	0.34	0.36	0.82	0.52	0.21	0.20	0.19	0.17
shell weight	3.47	3.45	2.53	3.21	2.56	2.85	4.90	3.34	**3.57**	**2.88**	1.67	1.84
(swt)	1.06	1.18	0.98	1.15	0.36	0.59	1.66	0.97	0.83	0.53	0.46	0.33
opercular	13.03	12.36	11.38	12.11	11.57	11.97	14.75	12.78	**11.79**	**10.88**	10.44	10.70
length (ol)	1.23	1.73	1.28	1.00	0.86	0.81	1.73	1.49	0.96	0.89	0.93	0.81
opercular	7.49	7.03	5.92	6.32	6.63	6.90	8.34	7.47	6.38	6.25	5.77	6.02
width (ow)	0.70	0.95	0.74	0.57	0.47	0.50	1.10	0.94	0.43	0.51	0.50	0.46
n =	37	14	16	13	17	16	19	17	13 swt=12	26 swt=22	17	10

($p<0.05$) for six of the variables (shell length and width, aperture length and width, shell weight and opercular length - Table 10).

B. sp. A

The differences in size between the sexes for both populations of *B.* sp. A (Groen River and Bakoven) were significant ($p<0.05$) for most of the variables, with the females being the larger (Table 11).

B. catarrhacta

For both populations of Kommetjie and Dalebrook, the mean size of the females was larger than the males (Table 12), and the differences were significant ($p<0.05$) for five of the variables at KM (including shell length) and eight of the variables at DK (including shell length and width).

B. sp. B

Most of the snails belonging to this species were collected from A-Frame, although one was found at Castle Rock, and a further two at Rooiels (see Table 1). The latter three animals were excluded from the analyses as they were smaller (shell lengths: 26.4, 28.2 and 30.0mm) than the A-Frame animals (minimum shell length: 31.6mm) and affected the mean values. They were however, used in a later discriminant analysis (see below for a comparison between *B. papyracea*, *B. pubescens* and *B.* sp. B). The means for the variables are given in Table 13. There was a significant difference ($p<0.05$) between the males and females only for spire height, although the females were slightly bigger for most measurements.

Table 11. Means and standard deviations for populations of *B*. sp A. The values in bold indicate significant differences between the sexes (m=males, f=females).

	Groen River (GR)		Bakoven (BO)	
	(m)	(f)	(m)	(f)
shell length	**37.36**	**41.03**	**32.46**	**37.60**
(sl)	2.86	1.46	3.65	2.98
shell width	**22.60**	**25.94**	**19.71**	**22.91**
(sw)	1.09	1.93	2.05	1.77
spire height	16.48	17.05	**13.27**	**16.31**
(sp)	2.20	2.27	1.68	1.88
aperture	**23.59**	**27.09**	**22.03**	**24.10**
length (al)	1.58	2.23	2.17	1.81
aperture	**10.86**	**13.15**	**9.80**	**11.51**
width (aw)	0.94	1.08	1.05	0.91
shoulder	**20.01**	**23.42**	**18.14**	**20.43**
height (ht)	1.25	2.63	2.03	1.67
thickness 1	0.98	0.99	**0.45**	**0.71**
(t1)	0.14	0.23	0.19	0.25
thickness 2	1.93	2.08	**1.07**	**1.42**
(t2)	0.41	0.47	0.41	0.51
body weight	**3.08**	**4.62**	**2.00**	**3.05**
(bwt)	0.66	1.28	0.66	0.68
shell weight	**6.76**	**8.89**	**3.34**	**5.18**
(swt)	1.47	2.54	1.44	1.46
opercular	**14.82**	**16.51**	**12.37**	**14.36**
length (ol)	1.67	1.72	1.57	1.42
opercular	**8.26**	**9.52**	**6.70**	**7.86**
width (ow)	0.75	0.94	0.78	0.73
n	11	12	14	22

Table 12. Means and standard deviations for populations of *B. catarrhacta*. Values in bold indicate significant differences between sexes (m=males, f=females).

	Kommetjie (KM)		Dalebrook (DK)	
	(m)	(f)	(m)	(f)
shell length	**25.06**	**28.70**	**29.29**	**32.87**
(sl)	3.21	3.33	2.25	2.34
shell width	13.93	15.56	**15.09**	**17.19**
(sw)	1.77	1.54	1.14	1.28
spire height	**11.29**	**13.25**	**14.10**	**15.93**
(sp)	1.33	1.47	1.24	0.90
aperture	15.36	17.22	**16.84**	**18.90**
length (al)	1.96	2.05	1.18	1.45
aperture	6.71	7.47	**7.35**	**8.47**
width (aw)	0.98	0.92	0.64	0.79
shoulder	**13.06**	**14.54**	**13.95**	**15.96**
height (ht)	1.59	1.64	1.04	1.35
thickness 1	0.27	0.30	0.37	0.41
(t1)	0.09	0.09	0.10	0.15
thickness 2	0.41	0.44	0.72	0.73
(t2)	0.15	0.14	0.18	0.17
body weight	**0.63**	**0.99**	**0.89**	**1.54**
(bwt)	0.22	0.30	0.20	0.37
shell weight	1.36	1.70	1.80	2.41
(swt)	0.56	0.54	0.47	0.68
opercular	**7.94**	**9.13**	**8.58**	**10.21**
length (ol)	0.96	0.77	0.72	1.08
opercular	4.40	4.87	4.79	5.53
width (ow)	0.49	0.47	0.44	0.64
n =	7	11	17	5

Table 13. Means and standard deviations for *B* . sp. B. Values in bold indicate significant differences between sexes (m=males, f=females).

	A-Frame (AF)	
	(m)	(f)
shell length	37.89	41.80
(sl)	2.27	3.99
shell width	19.75	21.00
(sw)	1.34	1.66
spire height	**17.64**	**20.83**
(sp)	1.05	2.19
aperture	22.23	22.83
length (al)	1.71	1.89
aperture	9.77	10.19
width (aw)	0.81	0.95
shoulder	18.43	19.95
height (ht)	1.03	1.78
thickness 1	0.56	0.48
(t1)	0.14	0.22
thickness 2	1.37	1.19
(t2)	0.36	0.36
body weight	2.23	2.72
(bwt)	0.45	0.92
shell weight	3.53	4.02
(swt)	0.87	0.93
opercular	14.44	15.25
length (ol)	1.44	1.72
opercular	7.49	7.87
width (ow)	0.40	0.70
n =	11	5

To summarize, in all of the species, there were significant differences between the sexes for most of the populations for at least one of the variables. There were only eight populations (in three species) where there were no significant differences for any of the variables. In all but one case (LAG-MB), where there were significant differences, the females were larger than the males.

Comparisons between populations within species

Tests of significance between the means for each of the variables between all populations were examined using analysis of variance. Due to the differences between the sexes, multiple pairwise comparisons, using Tukey's range test, were performed for each sex separately. For *B.* sp. A and *B. catarrhacta*, there being only two populations sampled, tests of significance were conducted using t-tests. The results of these tests are given in Table 14. Since all but the two shell thickness variables were highly correlated with shell length (ranging from 0.79 to 0.99 for all species), this variable can be used as a general indicator of size. The mean shell lengths for all of the populations are shown in Fig. 9.

B. papyracea

Analysis of variance indicated that for all variables, there was a significant difference ($p<0.001$) between the means of the four populations with sexes combined and separate. Tukey's tests of significance between population means (Table 14a) indicated that for both males and females, the CR population was significantly different ($p<0.01$) from the other three populations for most measurements of shell size and shell thickness. These snails were much smaller than those from the other three populations (Fig. 9). For both sexes AF was also significantly different from BB and BO for a number of size variables, and differed from the BO population in thickness. BB and BO only differed significantly in shell thickness (both sexes). In total, of the six between-population

Table 14. Tukey's studentized range tests for populations of *Burnupena*. The comparisons between sites were done separately for each sex. The variable names in the cells indicate a significant difference ($p<0.05$) between the two populations for that variable.

i) males

a) *B. papyracea*

	BB	BO	CR
BO	t1,2		
CR	s1-10 t1	s1-10 t1,2	
AF	s2,4,5,8	s2,4,5,8 t1,2	s1-10 t1,2

ii) females

	BB	BO	CR
BO	t1,2		
CR	s1-10	s1-10 t1,2	
AF	s3,4	s2-5,8 t1,2	s1-10 t1,2

KEY

sites	variables
GR = Groen River	s1 = shell length
BB = Blouberg	s2 = shell width
BO = Bakoven	s3 = spire height
OK = Oudekraal	s4 = aperture length
KM = Kommetjie	s5 = aperture width
CR = Castle Rock	s6 = shoulder height
AF = A-Frame	s7 = body weight
DK = Dalebrook	s8 =shell weight
SB = Sparks Bay	s9 = opercular length
RE = Rooi Els	s10 = opercular width
HM = Hermanus	
MB = Mossel Bay	t1 = shell thickness 1
PE = Port Elizabeth	t2 = shell thickness 2
DN = Durban	

i) males

b) *B. cincta*

	BB	OK	KM	CR	AF	DK	MB
OK	s2,5,7,9,10 t2						
KM		t2					
CR		s1,3,9,10					
AF		s1,3,7 t2					
DK	s5,9 t1	s7,10 t2					
MB	s5,9	s10 t2			s3		
PE	s5	t2		t2			

ii) females

	BB	OK	KM	CR	AF	DK	MB
OK	t2						
KM	s10 t1	t1,2					
CR	s1,3	s9,10 t2	s1,2,9,10 t1,2				
AF	s1,3	s3,7,9,10 t2	s1-4,7,9,10 t2				
DK	t1	s8 t2	s8,10	s1-4,6,8,9	s1-9		
MB		s8 t2	s10 t1	s1-3,8-10	s1-3,5,7,9	s4 t1	
PE	s5 t2	s8 t2		s1-6,8-10 t2	s1-10 t2		

i) males

c) *B. pubescens*

	CR	AF	RE
AF	s1-9		
RE			
PE	t2	s1-6 t1,2	

no comparisons were made between RE and the other three populations for t1 and t2

ii) females

	CR	AF	RE
AF	s1-9		
RE			
PE		s1,3,4,6	

e) *B.* sp. A

i) males

	GR
BO	s1-3,5-10 t1,2

ii) females

	GR
BO	s1,2,4-10 t1,2

i) males

d) *B. lagenaria*

	GR	BB	KM	DK	SB	HM	MB
BB							
KM	s2,4,5,7,9, s10 t1,2	s10 t1,2					
DK	s2,4-7,9 t1	t2	t2				
SB	s1,3,8-10	s3 t2	s1-10	s1-10			
HM	s5,7		s10 t2		s1-3,5-10		
MB	s2,4,5,7,10		t1,2		s1-7,9,10		
DN	s1-10	s1-10	s1-7 t1,2	s1-7,10	s1-10	s1-7,9,10	s1-8

ii) females

	GR	BB	KM	DK	SB	HM	MB
BB	s5,7,10						
KM	s2-5,7,10 t1,2	s3					
DK	s2,4-7,9,10						
SB	s1,3,8 t2	s1-3,7-10	s2,4,5,7-10	s2-10			
HM	s7	s3,10	s10 t1,2	s10	s7-9		
MB	s1,2,4-7,9, s10	s2,4,5,7	s1,3-7 t1,2	s4,5,7,10	s1-10	s1,2,4-7,9, s10	
DN	s1-10	s1-8	s1-8	s1-7,10	s1-10	s1-10	s1,3,6,7

f) *B. catarrhacta*

i) males

	KM
DK	s1,3,4,7 t1,2

ii) females

	KM
DK	s1,3,7-10 t2

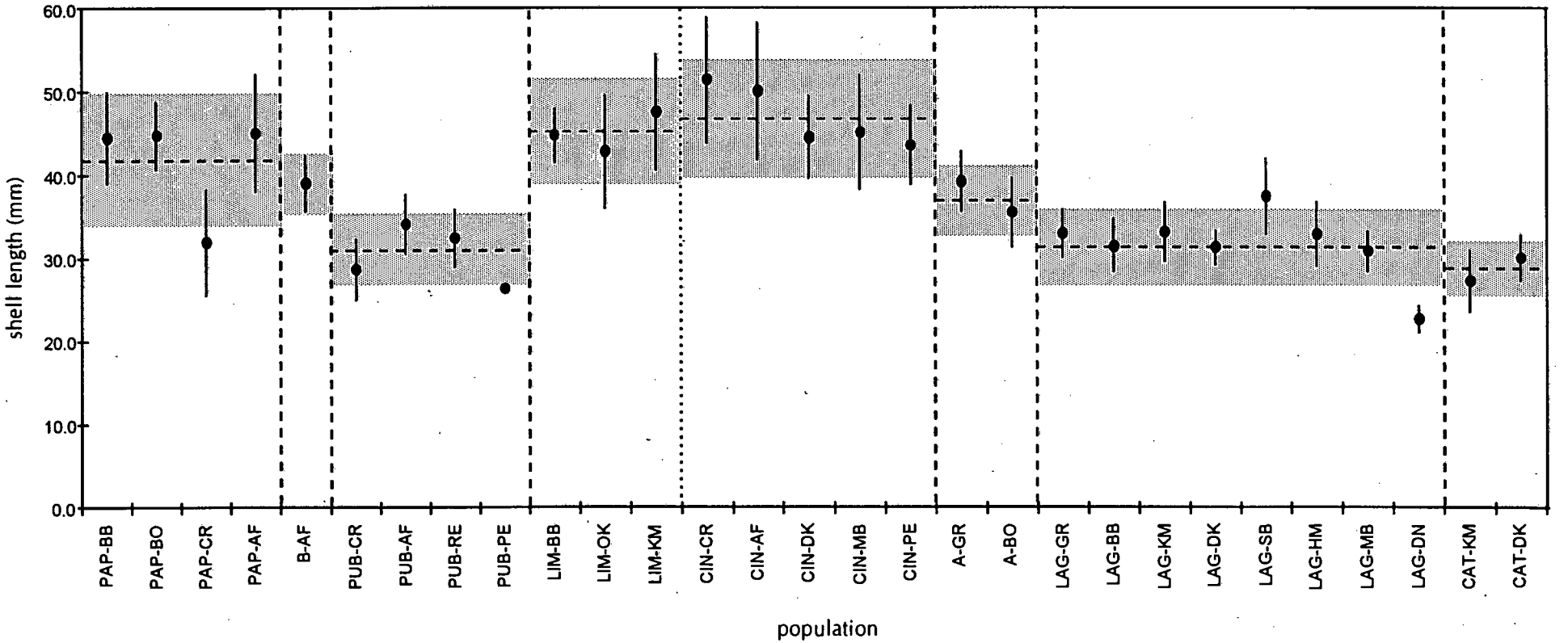


Figure 9. Mean and standard deviation of shell length for each population. The vertical dashed lines separate the species. The horizontal dashed lines and shaded areas indicate the mean and standard deviation for each species. See Table 1 for population abbreviations.

comparisons, five were significant, for both shell size and thickness, either between males or females or both.

B. cincta cincta and *B. cincta limbosa*

The analysis of variance indicated that for all variables, there were significant differences ($0.01 < p < 0.0001$) between the means of the eight populations (sexes combined and separate). Separate pairwise tests between sites were conducted for each sex (Table 14b: 28 tests each). For the males, there was a significant difference ($p<0.05$) in shell size in nine of the 28 comparisons, mostly involving the BB and OK populations: the OK males were smaller than the other populations (Table 8). Eight tests showed significant differences ($p<0.05$) for shell thickness (Table 14b), of which seven involved T2, the thickness of the shell taken at few mm in from the edge of the outer lip. The OK males were significantly thicker than most of the other populations for the latter variable.

There were many more significant tests between the females among the different sites, with 20 out of the 28 tests significant for shell size and 16 for shell thickness (Table 14b). This was probably due to the mostly larger sample sizes for the females (Table 8). The CR and AF populations differed significantly ($p<0.05$) from all other populations (except each other) for most of the shell size variables. These two populations had the largest animals (mean SL's of 53.9mm and 53.4mm respectively; Fig. 9). Sixteen tests showed significant differences ($p<0.05$) for shell thickness, of which only two were significant for both T1 and T2, and only four for T1 (Table 14b). As with the males, the OK population, which had much thicker shells (T2 = 3.0mm), was significantly different from all of the other female populations for T2. The PE population, the animals of which had the thinnest shells (T2 = 0.87mm - Table 8), were significantly different to three other populations (excluding OK).

B. pubescens

The mean size of the snails from CR, both male and female, was much smaller than that from AF (Fig. 9), as was the case in *B. papyracea*. Analysis of variance indicated that there was a significant difference ($p<0.01$) between the means of the four populations for all variables. Pairwise tests indicated that of the six between-population comparisons, two were significant for shell size and two for shell thickness. For both sexes, the AF population was significantly different ($p<0.05$) from CR and PE for shell size (Table 14c). Within the males, the PE individuals were significantly thinner ($p<0.05$) than both CR and AF individuals. No shell thickness (T1 and T2) differences were detected between the females.

B. lagenaria

An analysis of variance indicated that for all variables, there were significant differences ($p< 0.001$) between the means of the eight populations. Separate pairwise tests between sites were conducted for each sex (Table 14d : 28 tests each). For the males, there were 19 significant differences ($p<0.05$) in shell size. Seven of these involved the DN males, which were significantly smaller than all other populations for most of the size variables (Table 10; Fig. 9). The SB population also differed significantly from all other populations, having the largest snails (Table 10; Fig. 9). The GR population differed significantly from all populations excepting BB, these snails also being relatively large. There were nine significant differences ($p<0.05$) in shell thickness (T1 and T2), six of which involved the KM males, which had very much thinner shells (Table 10).

As with *B. cincta*, there were more significant tests between the females among the different sites, with 26 out of the 28 tests significant for shell size, although there were only four significant differences for shell thickness (Table 14d). The only comparisons where there were no significant differences in shell size, were between the DK and the BB and KM populations. The DN and MB populations had the smallest animals, whilst the SB females were larger (Table 10; Fig. 9).

Three of the four significant differences in shell thickness between the females involved the KM population, which had the thinnest snails (Table 10).

B. sp. A

T-tests showed that there were significant differences ($p < 0.01$) between the two populations for all of the variables. T-tests for each sex (Table 14e) indicated that there were significant differences in size ($p < 0.05$) and shell thickness ($p < 0.05$) between both males and females of the two populations. In both cases, the snails from GR were the largest (Fig. 9) and thickest (Table 11).

B. catarrhacta

T-tests indicated that there were significant differences between the two populations for seven of the 12 quantitative variables ($p < 0.05$), with the DK snails being bigger than those from KM (mean shell lengths of 30.1mm and 27.3mm respectively; Fig. 9). T-tests between sites (Table 14f) indicated significant differences for both shell size ($p < 0.05$) and shell thickness ($p < 0.05$) between the two populations for both sexes. The DK snails were larger and thicker than the KM snails for both sexes (Table 12).

Thus, in all of the species, except *B. catarrhacta*, there were significant differences between the means of the populations (sexes combined) for all of the 12 quantitative variables. In *B. catarrhacta*, only seven of the quantitative variables showed significant differences. For the pairwise tests, in all species there was at least one population (males or females) that was significantly different from at least one other population, for at least one size variable and one thickness variable. For the males, between 33% and 83% of the comparisons for size showed significant differences,

whilst for the females this ranged from 33% to 93%. For shell thickness, the number of significant comparisons ranged from 29% to 83% for the males, and from zero to 67% for the females.

Size-independent comparisons using ratios

The means of a series of ratios were calculated for each of the populations within each of the species (Appendix A). Ratios indicate relative size and shape, thus making it easier to compare populations, especially in those cases where mean population sizes differ. In all of the species the sexes were combined since the ratios were essentially the same, except for the body and shell weights relative to the shell length (bwt/sl, swt/sl): for most of the populations the females were heavier. In all of the species, differences between populations were observed, although for many of the ratios and most of the populations these differences were slight. The means of the ratios for all populations are shown in Fig. 10. The mean values shown below are for all populations in the species unless otherwise indicated.

B. papyracea

In this species the AF individuals were slightly narrower (sw/sl=0.49, $\bar{x}$ =0.52), with a longer spire (sp/sl=0.53, $\bar{x}$ =0.50) and a shorter aperture (al/sl=0.51, $\bar{x}$ =0.55) than animals in the other three populations (Fig. 10a-b). The BO animals were much thicker-shelled (t1/sl=0.017, $\bar{x}$ =0.013; t2/sl=0.045, $\bar{x}$ =0.032) than the others, which were all similar (Fig. 10c). In terms of relative shell and body weight (Fig. 10d), the CR population was very much lighter than the other three populations (bwt/sl=0.042; swt/sl=0.060), being just over half the relative weight of the AF individuals (bwt/sl=0.079; swt/sl=0.110), and less than half of the relative weight of the BO and BB individuals (bwt/sl=0.092; swt/sl=0.146 and bwt/sl=0.093; swt/sl=0.136 respectively). Both the males and females showed the same trend.

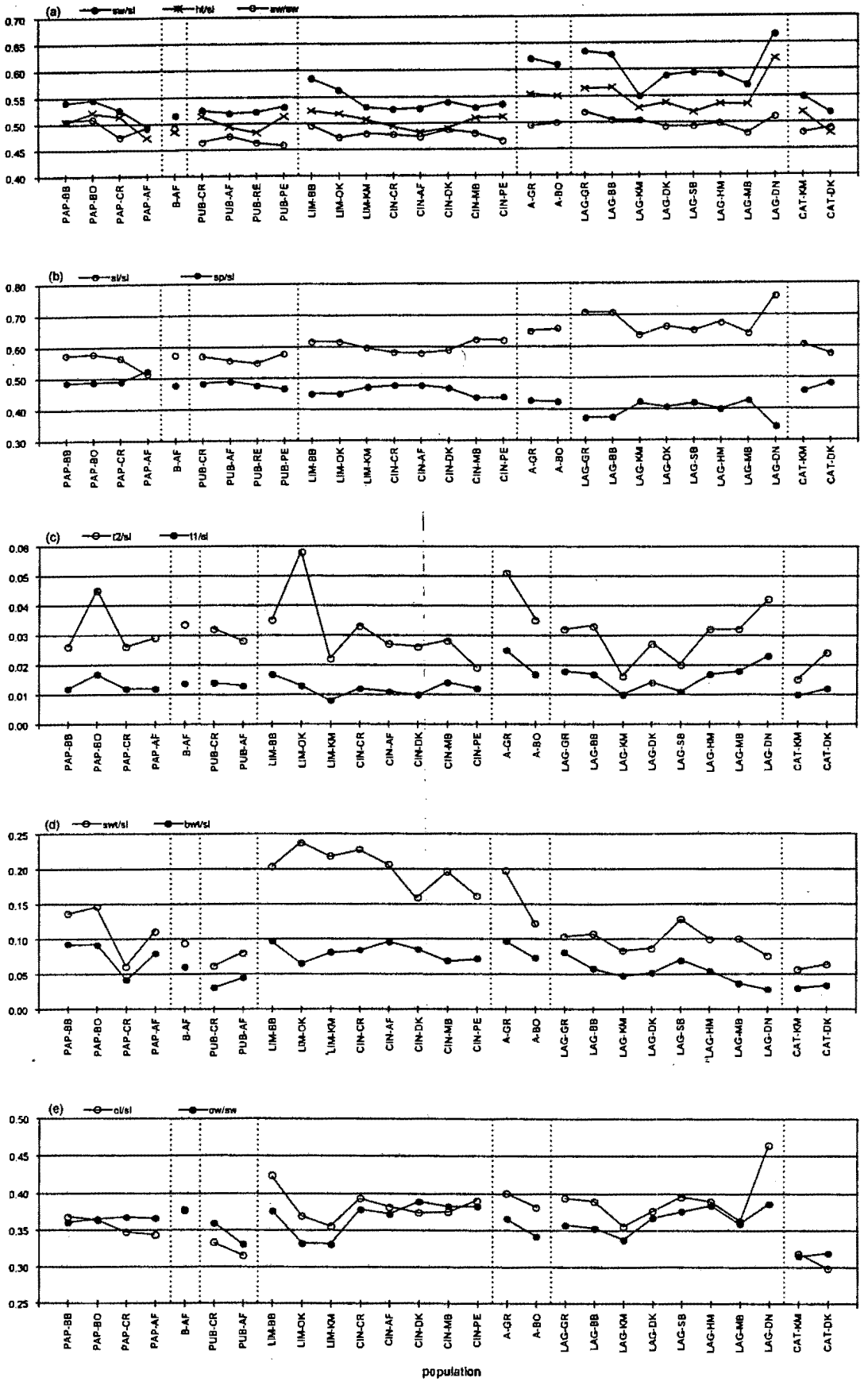


Figure 10. Means of ratios for each of the populations of *Burnupena* sampled. Shell length was used as the standard demoninator except for aperture and operculum widths where shell width was used. The lines connecting the points have been included as a visual aid only. The dotted vertical lines separate the species. (a) shell width, shoulder height and aperture width; (b) aperture length and spire height; (c) shell thickness 1 and 2; (d) shell and body weights; (e) operculum length and width.

B. cincta cincta and *B. cincta limbosa*

There were few differences between the populations of *B. cincta*. The BB and OK populations (both *B. c. limbosa*) tended to have the widest snails (sw/sl=0.59 and 0.56 respectively, $\bar{x}$ =0.54 for all populations, Fig. 10a), and the highest shoulder (ht/sl=0.52, $\bar{x}$ =0.50, Fig. 10a). The other *B. c. limbosa* population, KM, was more similar to the three *B. c. cincta* populations from the Western Overlap (CR, AF and DK), being narrower, and with a longer spire (sp/sl=0.47, $\bar{x}$ for Western Overlap populations=0.47, $\bar{x}$ for BB and BO=0.45, Fig. 10b). The two *B. c. cincta* South Coast populations, MB and PE were similar to the Western Overlap populations in terms of width (sw/sl=0.53 and 0.54 respectively), but were more similar to BB and OK in terms of spire height (sp/sl=0.44 for both populations). The OK shells were thickest (t2/sl=0.058, overall $\bar{x}$ =0.031, Fig. 10c) and heaviest (swt/sl=0.237, overall $\bar{x}$ =0.201, Fig. 10d), but also had the lightest body weights (bwt/sl=0.065, overall $\bar{x}$ =0.081, Fig. 10d).

B. pubescens

The results for *B. pubescens* indicated that the populations were fairly uniform in their relative proportions, but that the RE and PE populations had much thinner shells (t1/sl=0.009; t2/sl=0.018 and 0.016 respectively, Fig. 10c) than those of snails from AF and CR (t1/sl=0.013 and 0.014 respectively; t2/sl=0.028 and 0.032 respectively).

B. lagenaria

In this species the DN and KM populations were usually the most different. The DN snails were the widest (sw/sl=0.67, $\bar{x}$ =0.61, Fig. 10a), had the highest shoulder (ht/sl=0.62, $\bar{x}$ =0.55, Fig. 10a), and largest operculum (ol/sl=0.46, $\bar{x}$ =0.39; ow/sw=0.39, $\bar{x}$ =0.32, Fig. 10e). They had

much shorter spires (sp/sl=0.34, $\bar{x}$ =0.40, Fig. 10b). They were the thickest (t1/sl=0.023, $\bar{x}$ =0.016; t2/sl=0.042, $\bar{x}$ =0.029, Fig. 10c), but had the lowest shell weights (swt/sl=0.075, $\bar{x}$ =0.096, Fig. 10d) as well as body weights (bwt/sl=0.028, $\bar{x}$ =0.053, Fig. 10d). The KM snails tended to be narrower (sw/sl=0.55, $\bar{x}$ =0.61, Fig. 10a), with the thinnest shells (t1/sl=0.010, $\bar{x}$ =0.016; t2/sl=0.016, $\bar{x}$ =0.029, Fig. 10c). The SB snails had the heaviest shells (swt/sl=0.128, $\bar{x}$ =0.096, Fig. 10d) but, paradoxically, had very thin-lipped shells (t1/sl=0.011, $\bar{x}$ =0.016; t2/sl=0.020, $\bar{x}$ =0.029, Fig. 10c), only KM shells being thinner.

B. sp. A

The two populations were similar for most ratios except for shell thickness (Fig. 10c) and shell and body weights (Fig. 10d). The GR snails were thicker than those from BO (t1/sl=0.025 and 0.017 respectively; t2/sl=0.051 and 0.035 respectively), and also heavier (swt/sl=0.197 and 0.122 respectively; bwt/sl=0.097 and 0.073 respectively).

B. catarrhacta

For *B. catarrhacta*, the KM snails were wider (sw/sl=0.55 and 0.52 for DK, Fig. 10a), with a shorter spire (sp/sl=0.46 and 0.48, Fig. 10b), a longer aperture (al/sl=0.61 and 0.58, Fig. 10b), and a higher shoulder (ht/sl=0.52 and 0.48, Fig. 10a) than the DK snails. They also were much thinner shelled (t2/sl=0.015 and 0.024, Fig. 10c).

Qualitative variables

The means and standard deviations of the six qualitative variables for each of the populations are given in Appendix B. Frequency histograms of the different states for each of these

variables, and for all populations, are shown in Fig. 11. For the most part, the frequency distributions of the different states within each of the populations reflect the distribution for the species as a whole (Fig. 5). There are however, a few points worth noting.

The bimodality of the two rib variables for *B. papyracea*, as previously noted, is obvious (Fig. 11a-b): the two West Coast populations having smooth shells with no ribs, whilst the Western Overlap populations have strongly ribbed shells with an intermediate number of ribs. The differences between the West Coast *B. cincta limbosa* (LIM) and the Western Overlap and South Coast populations of *B. cincta cincta* (CIN) for rib strength (RIB1) can also clearly be seen. What is also apparent, is that the two South Coast populations (MB and PE) between them tended to have more weakly ribbed shells, whilst the Western Overlap populations generally had strongly ribbed shells. For *B. lagenaria* as a whole, the histogram in Fig. 5a showed almost equal number of the two intermediate rib categories (smooth/weak and weak). However, only three of the eight populations showed clear variations for RIB1 (Fig. 11a), with most individuals in the other five populations tending to be similar. There were no regional trends in rib strength for this species.

Eight of the 29 populations had some individuals that were smooth and others that were ribbed (RIB2; Fig. 11b), namely the three *B.* c. *limbosa* populations, four of the eight *B. lagenaria* populations and one *B. catarrhacta* population. The number of ribs was highly consistent, all individuals in all of the populations within a species having the same number of ribs.

The histogram for constriction (Fig. 11c) showed that the small proportion of *B. pubescens* individuals having a marked constriction (Fig. 5c), were all collected from PE. The populations of *B. cincta* showed regional differences in two of the constriction states, with a slight constriction being more frequent in the West Coast (LIM) populations, replaced by a marked constriction in the Western Overlap and South Coast (CIN) populations. Most individuals in most of the populations however, had a moderate constriction. This pattern was repeated in *B. lagenaria*, with only the three West Coast populations having individuals with a slight constriction, although snails with a marked constriction were also found in the West Coast region (unlike *B. cincta*).

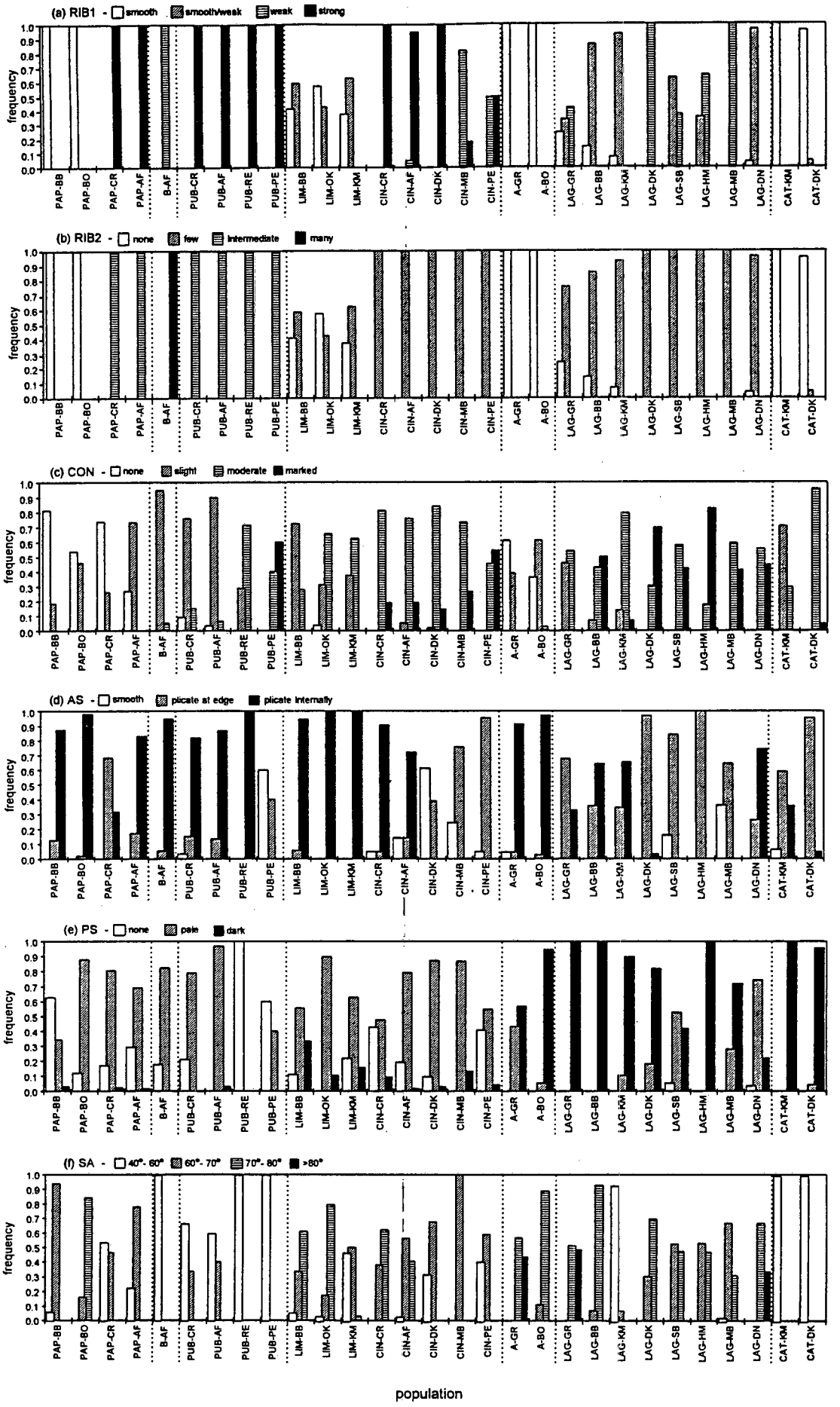


Figure 11. Frequency histograms for the categories of the qualitative variables. The vertical dotted lines separate the species. (a)strength of ribs (RIB1), (b) number of ribs (RIB2), (c) constriction (CON), (d) aperture sculpture (AS), (e) parietal scar (PS) and (f) spire angle (SA).

There were three states for aperture sculpture (Fig. 11d). As with constriction, virtually the only *B. pubescens* population to have individuals with a smooth shell internally was the PE population. The situation for *B. cincta* was interesting, with the five most westerly populations having an internally plicate shell as the most common state, almost to the exclusion of the other two states, whereas this state was completely absent in the three most easterly populations. The distribution of this state (plicate internally) was disjunct in *B. lagenaria*. It was common in four of the eight populations, the three West Coast populations and the DN population, but near absent in the other four populations.

In relation to the parietal scar, the species were divided into two groups as previously noted (Fig. 5e). The results for each of the populations within a species (Fig. 11e) were similar to those obtained for the species as a whole.

The angle of the spire was fairly variable within most of the species (Fig. 11f). The only individuals within *B. papyracea* to have shells with a spire angle greater than 70°, were from BO. Apart from the KM population, a regional trend in spire angle for *B. cincta* was apparent. The most westerly populations tended to have more individuals with relatively obtuse spires, whilst in the three easterly populations no individuals had spire angles greater than 70°. The KM population had snails with relatively acute spires. The same was true for the *B. lagenaria* KM population, which, apart from one MB snail, was the only population to have snails with spire angles less than 60°. Furthermore, only two of these KM individuals had shells with a spire angle greater than 60°. Only the two geographically extreme populations of *B. lagenaria* had individuals with very obtuse spires (greater than 80°).

In summary, the within species variation was generally high, there being no variation within a species in only three of the species, and these in three of the variables: *B. pubescens* and *B.* sp. A were both invariant for both RIB1 and RIB2, and *B. catarrhacta* was invariant for spire angle. Across all populations, within-population variation was lowest for RIB2 followed by RIB1, with 21

and 16 of the 29 populations respectively, being invariant. *B.* sp. B was very uniform across all of the qualitative variables, being invariant for three of them, and for the other three, the frequency of the most common state was not less than 0.8.

Classification of individuals by means of discriminant function analysis

In the discriminant analyses, all 18 variables were entered for analysis, with the following exceptions: shoulder height was omitted for *B. cincta*; body and shell weights, and opercular length and width were excluded for *B. pubescens* since these data were missing for the RE and PE populations. The variables are analysed in a stepwise manner by the discriminant analysis, and those variables that do not contribute to the discrimination are discarded. The variables employed by the discriminant analysis were subsequently used in the canonical variate analyses. The results of the jackknifed classifications for each of the species are shown in Table 15.

B. papyracea

Eight of the 18 variables were employed in the discriminant analysis. These were spire, aperture length and width, shell thickness 2, body and shell weights, height and spire angle. The jackknifed classification function correctly identified 100% of the AF population, 90% of the CR population, 88% of the BO population and 97% of the BB population (Table 15), with an overall total of 93% correctly identified.

The analysis was repeated with the males and females as separate groups. Here the percentage of cases that were identified to the correct sex and population ranged from 43% to 77% with the overall total at 64% (Table 15). These values were much lower than when the sexes were pooled. However, nearly three times as many of the misallocations were between males and

Table 15. Jackknifed classification of the discriminant analysis showing the percentage of snails allocated to the correct population for each of the species. The number in parentheses is the number of individuals from each population. The total percentage allocated to the correct sex, obtained running the analysis with sexes separately, is also shown.

Site (abbrev.)		Species[1]						
		PAP	CIN/LIM	CIN/LIM[2]	PUB	LAG	A	CAT
Groen River	(GR)					90.9 (33)	73.9 (23)	
Blouberg	(BB)	96.7 (30)	94.1 (17)	88.9 (18)		78.6 (14)		
Bakoven	(BO)	88.4 (43)					88.9 (36)	
Oudekraal	(OK)		96.3 (27)	96.4 (28)				
Kommetjie	(KM)		96.9 (32)	96.9 (32)		100 (28)		88.9 (18)
Castle Rock	(CR)	90.0 (40)	76.2 (21)	71.4 (21)	84.8 (33)			
A-Frame	(AF)	100 (30)	70.0 (40)	65.0 (40)	90.0 (20)			
Dalebrook	(DK)		96.7 (60)	96.7 (60)		78.1 (32)		100 (21)
Sparks Bay	(SB)					93.3 (15)		
Rooiels	(RE)				100 (4)			
Hermanus	(HM)					64.7 (17)		
Mossel Bay	(MB)		90.0 (40)	87.5 (40)		93.8 (32)		
Port Elizabeth	(PE)		63.6 (22)	68.2 (22)	100 (5)			
Durban	(DN)					100 (25)		
total to correct population		93.0	86.9	85.4	88.7	88.8	83.1	94.9
total to correct sex		64.3	64.5			67.9	72.9	84.6

[1]species abbreviations as in Table 3
[2]excluding the two rib variables

females of the same population than between different populations. This implies that although there are differences between the sexes, these differences are not as great as the differences between sites.

B. cincta cincta and *B. cincta limbosa*

Thirteen of the 17 variables were employed in the stepwise discriminant analysis (shell length and width, constriction and parietal scar were discarded). The jackknifed classification correctly classified about 87% of the individuals to their correct population, with the percentages ranging from 64% correct for PE up to 97% for KM and DK (Table 15). Apart from the two South Coast populations (MB and PE), the individuals that were misidentified were always placed into populations in the same region. The MB and PE populations had individuals allocated to all/some of the Western Overlap populations (CR, AF and DK), but not to the West Coast populations (BB, OK and KM).

As was found with *B. papyracea*, the analysis with males and females as separate groups (excluding BB and PE for which there was no difference between the sexes), showed that the overall percentage of correct classifications was lower (65%). Of the cases that were incorrectly allocated, almost 60% were between males and females of the same population (Table 15).

The discriminant analysis was repeated for *B. cincta* (Table 15), but this time the two rib variables were excluded, to establish whether other morphometric differences could be detected between *B. c. cincta* and *B. c. limbosa* populations. Of the 15 variables used, 13 were entered into the stepwise discriminant analysis (shell width and aperture length being omitted). The overall percentage of correctly identified individuals was about 85% (Table 15), only slightly lower than when RIB1 and RIB2 were used. None of the Western Overlap and South Coast populations were allocated to the West Coast as before, but a few individuals from BB (West Coast) were allocated to the Western Overlap populations. Four populations, namely BB, CR, AF and MB, had slightly lower

percentages of correctly identified snails; three, (OK, KM and DK), were unchanged; one (the PE population), showed an increase in the number of correctly identified snails (Table 15).

B. pubescens

Seven of the fourteen variables were selected by the analysis: shell width, spire height, shoulder height, shell thickness 2, constriction, aperture sculpture and parietal scar. The jackknifed classification function correctly identified approximately 85% of the CR population, 90% of the AF population, and 100% of the RE and PE populations (Table 15). An analysis with the sexes kept separate was not performed on this species, since only the CR population showed significant differences between sexes for overall size.

B. lagenaria

Four variables, shell length and width, aperture width and shell thickness 1, were not used in the discriminant analysis. The jackknifed classification correctly classified about 89% of the individuals to their correct population, with the percentages ranging from 65% correct for HM up to 100% for KM and DN (Table 15). A second analysis with the sexes separated for four of the populations gave an overall classification of only 68% (Table 15), with values ranging from 47% to 93%. As found previously, a large portion (67%) of the misclassified individuals were to the opposite sex of the same population.

B. sp. A

Only three of the 18 variables, shell thickness 1, parietal scar and spire angle, were found to be useful in separating the two populations of *B.* sp. A. The jackknifed classification correctly

identified about 74% of the GR individuals, and about 89% of the BO individuals (Table 15). With the sexes separate, the jackknifed classification correctly identified about 73% of the individuals to the correct population and sex (Table 15), the percentages ranging from 64% to 77%. As with other species, most of the misclassifications (almost 70%) were to the opposite sex but the correct population.

B. catarrhacta

In the first discriminant analysis of the two populations of *B. catarrhacta* (Table 15) a total of about 95% of the individuals were correctly identified (89% for KM and 100% for DK). Six variables, shoulder height, shell thickness 2, body weight, number of ribs, constriction and aperture sculpture were employed in the discriminant analysis to discriminate between the two populations. In a second analysis with the sexes separate, between 64% and 100% of the individuals were classified to the correct group, with the total at 85% (Table 15). Only one individual was placed into the wrong population, with the remaining misallocations going to the wrong sex.

To summarize, viewing all of the species, between 83% and 95% of the individuals were allocated to the correct population. When the analysis was performed with the sexes as separate groups, between 67% and 89% of those individuals that were misclassified, were allocated to the opposite sex of the correct population.

Separation of the populations by means of canonical variate analysis

Plots were produced of the first and second canonical variables for each of the species, except for *B.* sp. A and *B. catarrhacta*. Since there were only two populations of each of these

species, a histogram was plotted for the single canonical variable calculated. The standardized coefficients of the canonical variates for each of the species are given in Table 16, together with the proportion of the total variance accounted for by the canonical variables.

B. papyracea

A plot of the first two canonical variables (Fig. 12) shows no overlap between the two West Coast populations (BO and BB) and the two Western Overlap populations (AF and CR). The former two populations overlap partially, whilst the latter two populations only overlap slightly. The standardized coefficients of the canonical variables are given in Table 16. These coefficients indicate that the separation between the populations along the first axis is achieved mainly by a contrast of shell weight with spire length and shoulder height. Separation along the second axis was mostly due to differences in spire length and shell weight. About 82% of the total variability within these variables was accounted for by these first two canonical variables. Two of the variables, rib strength and number of ribs, could not be used in the discriminant analysis because they were invariant in all four populations (Fig. 11a-b). However, they can be used to separate the West Coast from the Western Overlap populations as described above. The West Coast snails do not have the fairly strong spiral ribs found in the Western Overlap snails.

B. cincta cincta and *B. cincta limbosa*

Figure 13a shows the plot of the first two canonical variables, and their standardized coefficients are given in Table 16. These indicate that the variable RIB1 (the strength of the ribs) contributed the most to the separation between the populations along the first axis, and that RIB2 (no. of ribs), shell weight and opercular width were also important. There is a clear separation between the three West Coast *B. c. limbosa* populations and the Western Overlap and South Coast *B. c. cincta* populations along the first axis, which accounted for about 72% of the variance. Within

Table 16. Standardized coefficients for the canonical variables in each of the species. Only one canonical variable was calculated for *B*. sp. A and *B. catarrhacta*, since in both species only two populations were sampled.

	Species[1]											
	PAP		CIN/LIM		CIN/LIM[2]		PUB		LAG		A	CAT
variable	cv 1	cv 2	cv 1	cv 2	cv 1	cv 2	cv 1	cv 2	cv 1	cv 2	cv 1	cv 1
shell length					**1.26**	**2.99**						
shell width							**-1.82**	**2.46**				
spire height	**-2.51**	**2.27**	0.25	**1.85**	-0.29	-0.15	0.76	0.89	0.76	0.57		
aperture length	1.48	0.71	-0.47	**1.11**					0.74	0.58		
aperture width	1.13	0.23	0.32	-0.30	0.03	-0.63						
shoulder height	**-2.09**	-0.30					0.49	**-2.26**	-0.25	-0.41		**-2.15**
shell thickness 1			0.35	-0.29	-0.31	0.01					**-0.65**	
shell thickness 2	-0.59	0.81	0.01	0.44	0.01	0.89	**1.16**	-0.66	-0.22	0.69		1.08
body weight	0.74	-0.58	0.17	**-1.25**	-0.22	**-2.81**			**1.77**	-0.32		1.53
shell weight	**2.05**	**-2.29**	**-1.00**	-0.15	**1.63**	**-1.69**			-0.42	-0.41		
opercular length			-0.16	0.36	0.22	0.57			-0.70	**-0.91**		
opercular width			**0.76**	**-1.56**	**-2.31**	**1.10**			**-1.22**	0.72		
rib strength			**1.55**	0.40					0.04	**1.00**		
no. ribs			**-1.02**	-0.24					-0.16	-0.73		0.57
constriction					-0.24	0.15	-0.65	-0.12	-0.16	-0.03		0.70
aperture sculpture			-0.17	0.50	0.59	0.03	0.47	0.18	0.07	-0.52		-0.97
parietal scar					0.17	0.14	0.36	0.19	0.45	0.20	0.49	
spire angle	-0.19	0.67	0.16	0.63	0.22	0.79			0.03	0.40	**-0.66**	
cumulative % of total variance	56.3	81.7	72.3	82.9	44.1	63.5	61.6	91.4	45.5	68.6	100	100

The values in bold indicate the variables that contribute most to the separation of the populations in each of the species.

[1]species abbreviations as in Table 3

[2]excluding the two rib variables

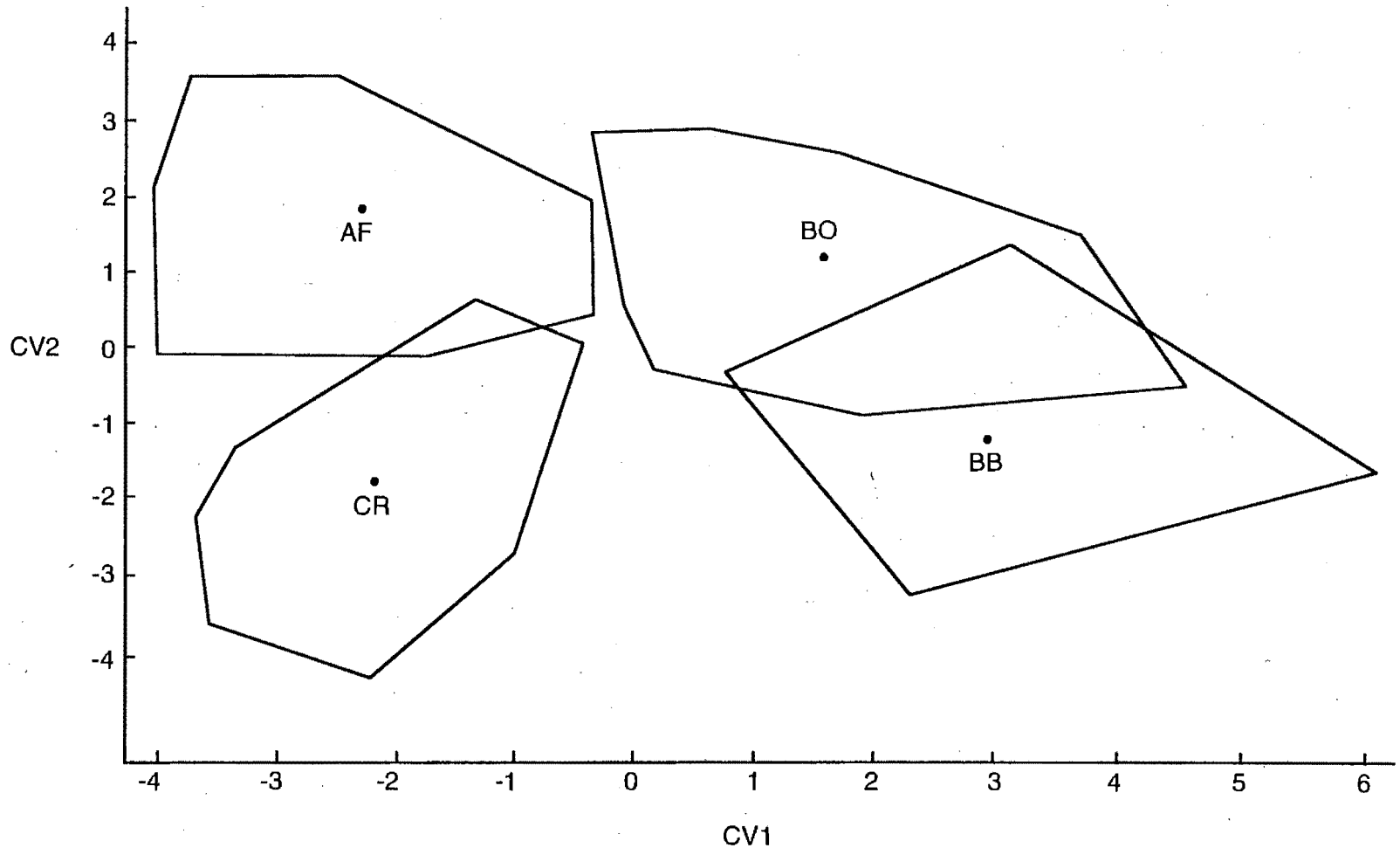


Figure 12. Plot of the first two canonical variables for the populations of *B. papyracea*.

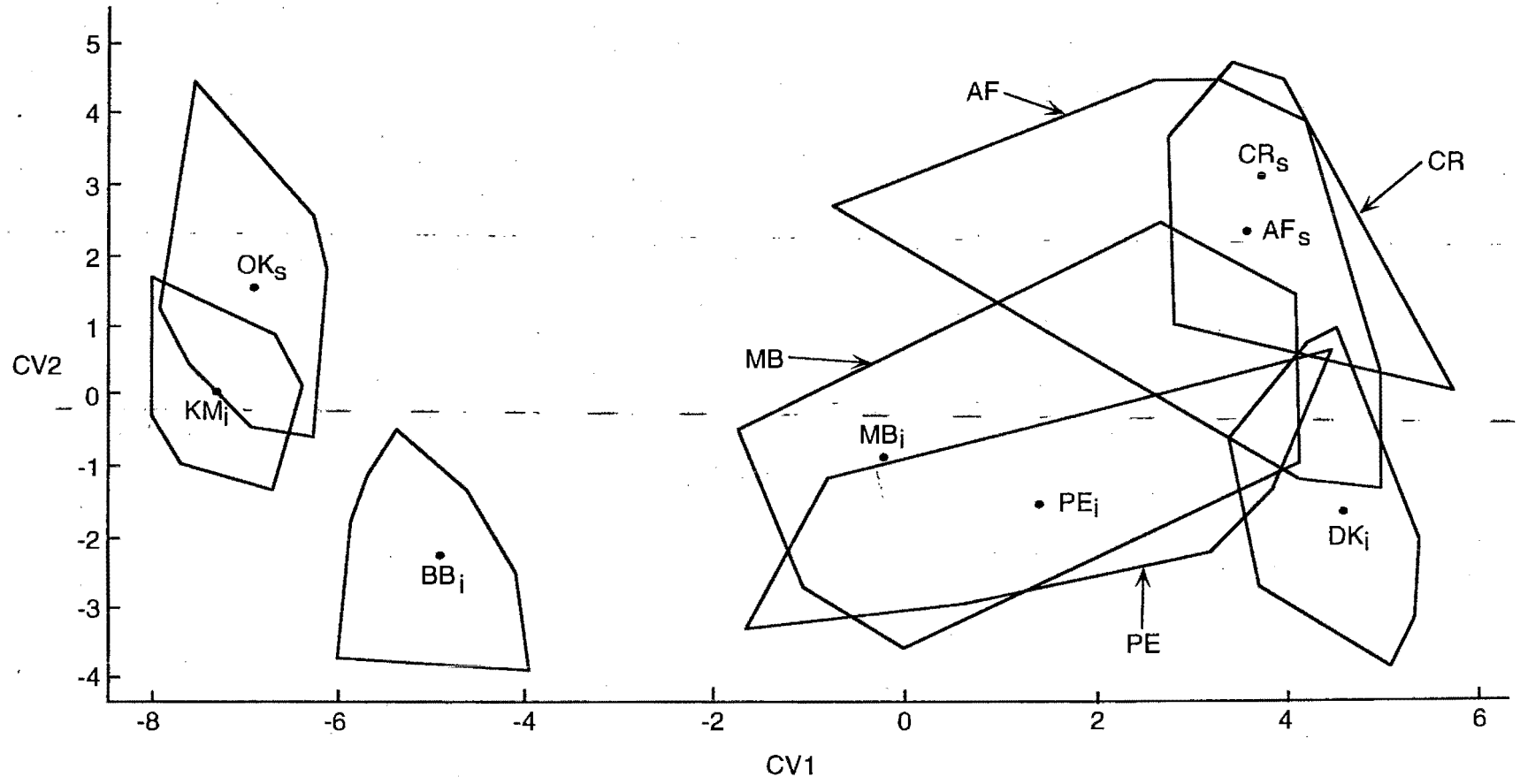


Figure 13a. Plot of the first two canonical variables for the populations of *B. cincta*. The outlines are convex hulls surrounding all of the points for that population. The abbreviations for the centroids of each population are given in Table 8. The subscript following the population abbreviation indicates whether the population was collected subtidally (s) or intertidally (i).

the West Coast, the OK and KM populations were well separated from the BB population. The first axis was unable to differentiate the three Western Overlap and two South Coast populations, although they were partially separated into two groups along this axis (high and medium values for CV1 respectively), which reflected their values for RIB1 (means for CR, AF and DK were 4.0, 3.95 and 4.0 respectively; means for MB and PE were 3.18 and 3.50; Appendix B). There was some separation between the populations along the second axis which accounted for a further 10% of the variance. The variables contributing most to this separation were spire, aperture length, opercular width and body weight (Table 16). The OK and KM populations showed partial separation, as did most of the Western Overlap and South Coast populations, except for AF and CR (Fig. 13a). The habitats of the populations are indicated on Fig 13a, and there appears to be some separation of the subtidal (AF, CR and OK) and intertidal populations along the second axis. Although some of the variables contribute more to the separation of the populations than others, the other variables are not without effect, and it is not obvious from the data (Figs 9, 10 and 11) which variables are responsible for this separation. Further, the variables causing the separation of the AF and CR populations from the other Western Overlap and South Coast populations, may be different to those responsible for the separation of the OK population.

As with the discriminant analysis, the canonical analysis was repeated, but this time the two rib variables were excluded. A plot of the first two canonical variables is shown in Fig. 13b, and the variables that contributed most to the separation between the populations are indicated in Table 16. Two of the *B. c. limbosa* populations, OK and KM, could still be separated from the *B. c. cincta* populations, but the BB population overlapped substantially with the other populations (Fig. 13b). The variables responsible for most of the separation of the two *B. c. limbosa* populations were operculum width and shell weight. OK and KM snails tended to have relatively narrower opercula (ow/sw = 0.33 relative to the mean for the species, 0.37) and heavier shells (swt/sl = 0.237 and 0.218 - species mean = 0.201).

B. pubescens

A plot of the first two canonical variables, accounting for over 90% of the total variance (Fig. 14), showed the complete separation of the RE and PE populations, and a small overlap of the AF and CR populations. The variables that contributed the most towards the separation of the populations were shell width and shell thickness 2 for the first canonical variable, and shell width and shoulder height for the second canonical variable (Table 16).

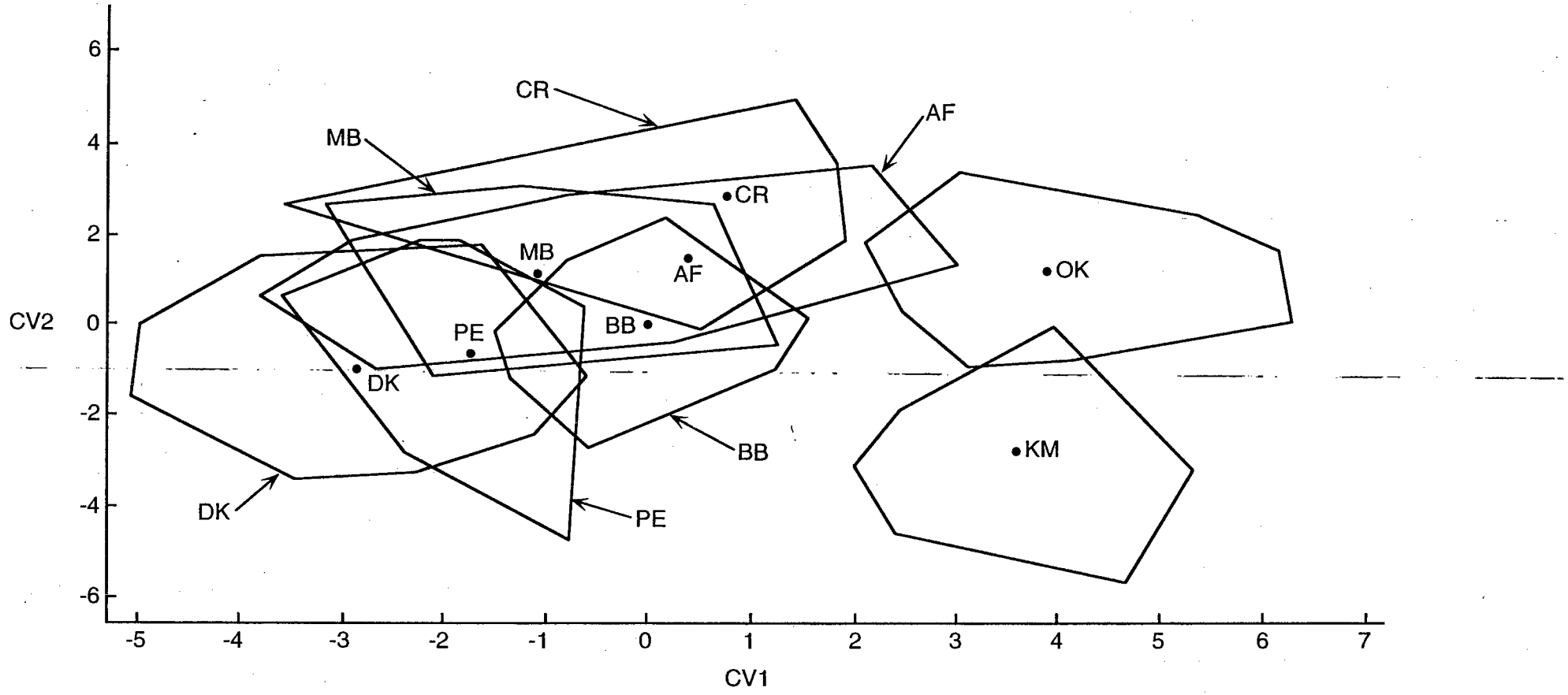


Figure 13b. Plot of the first two canonical variables for the populations of *B. cincta*, with the two RIB variables excluded. The outlines are the convex hulls surrounding all of the points for each population. The abbreviations for the populations are given in Table 8.

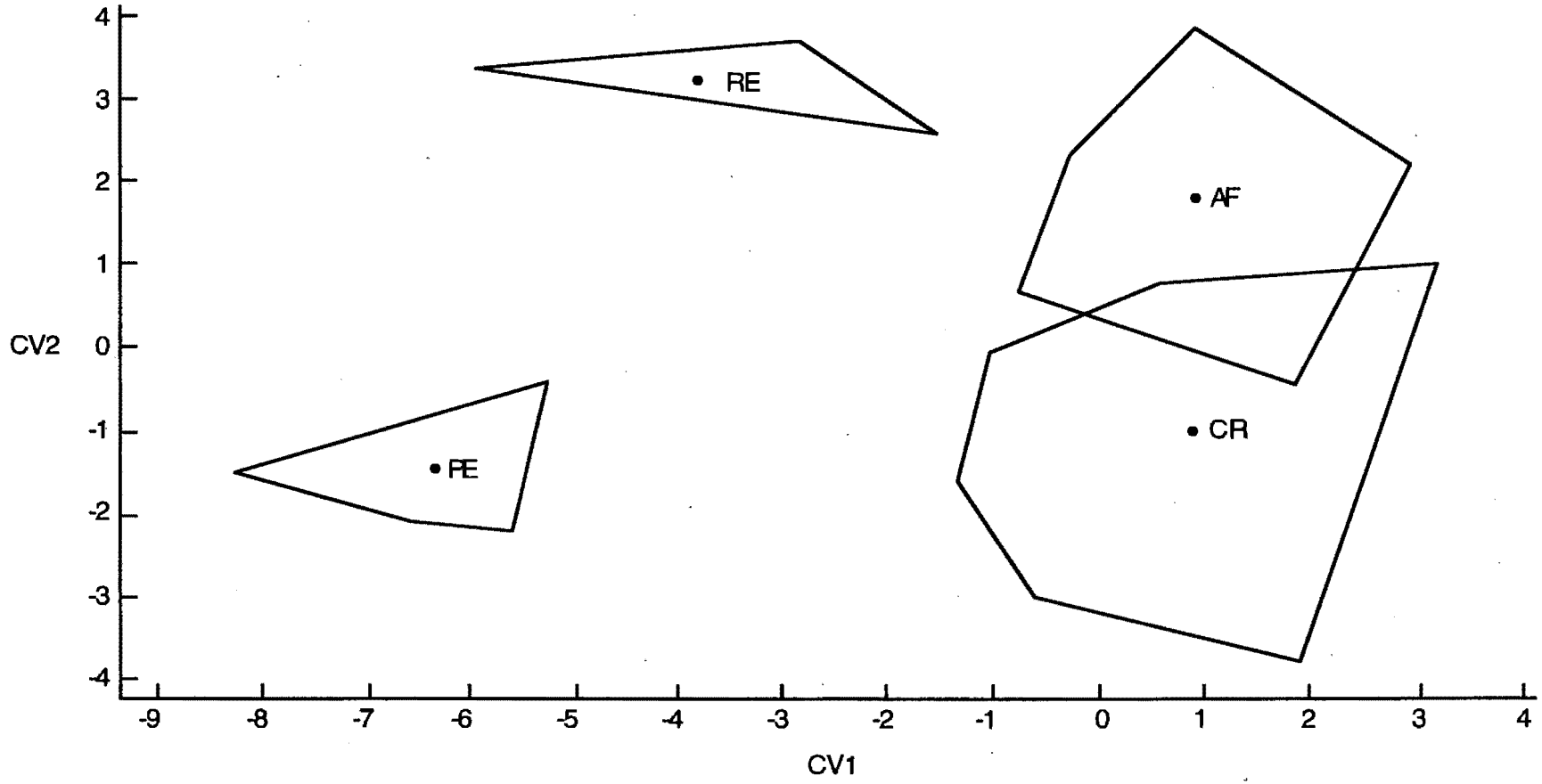


Figure 14. Plot of the first two canonical variables for the populations of *B. pubescens*.

B. lagenaria

The standardized coefficients for the canonical variables (Table 16) indicate that separation between the populations along the axis of the first canonical variable was mainly due to the contrast of body weight with opercular width. The variables contributing most to the separation of the populations along the second axis were rib strength and opercular length. The plot of canonical variables 1 and 2 is shown in Fig. 15. Together they account for just under 70% of the total variance. There was complete separation of the DN population from the remaining seven populations along the first axis. The KM population was also completely separated from the other six populations, most of which overlapped substantially. Ignoring the DN population, the overall pattern that emerged from Fig. 15 was one of a general trend from the South Coast population (MB), which had a higher CV2 and lower CV 1, through the three Western Overlap populations (DK, SB and HM), and then to the three West Coast populations (GR, BB and KM) which had lower CV2's and higher CV1's. There was almost no overlap between the South Coast and West Coast groups.

B. sp. A and *B. catarrhacta*

Only one canonical variate was calculated for each of these two species, using only three and six variables respectively (Table 16). The two histograms produced showed some overlap between the two *B.* sp. A populations (Fig. 16), but there was complete separation between the two *B. catarrhacta* populations (Fig. 17).

In summary, between three and fourteen of the 18 variables were found to be useful in discriminating between the populations within a species. However, no single variable was used to discriminate between populations in all of the species. One variable, shell thickness 2, was used in five of the species, whilst five of the variables were used in four of the species. Considering only those variables deemed to contribute the most to the separation between the populations, shoulder

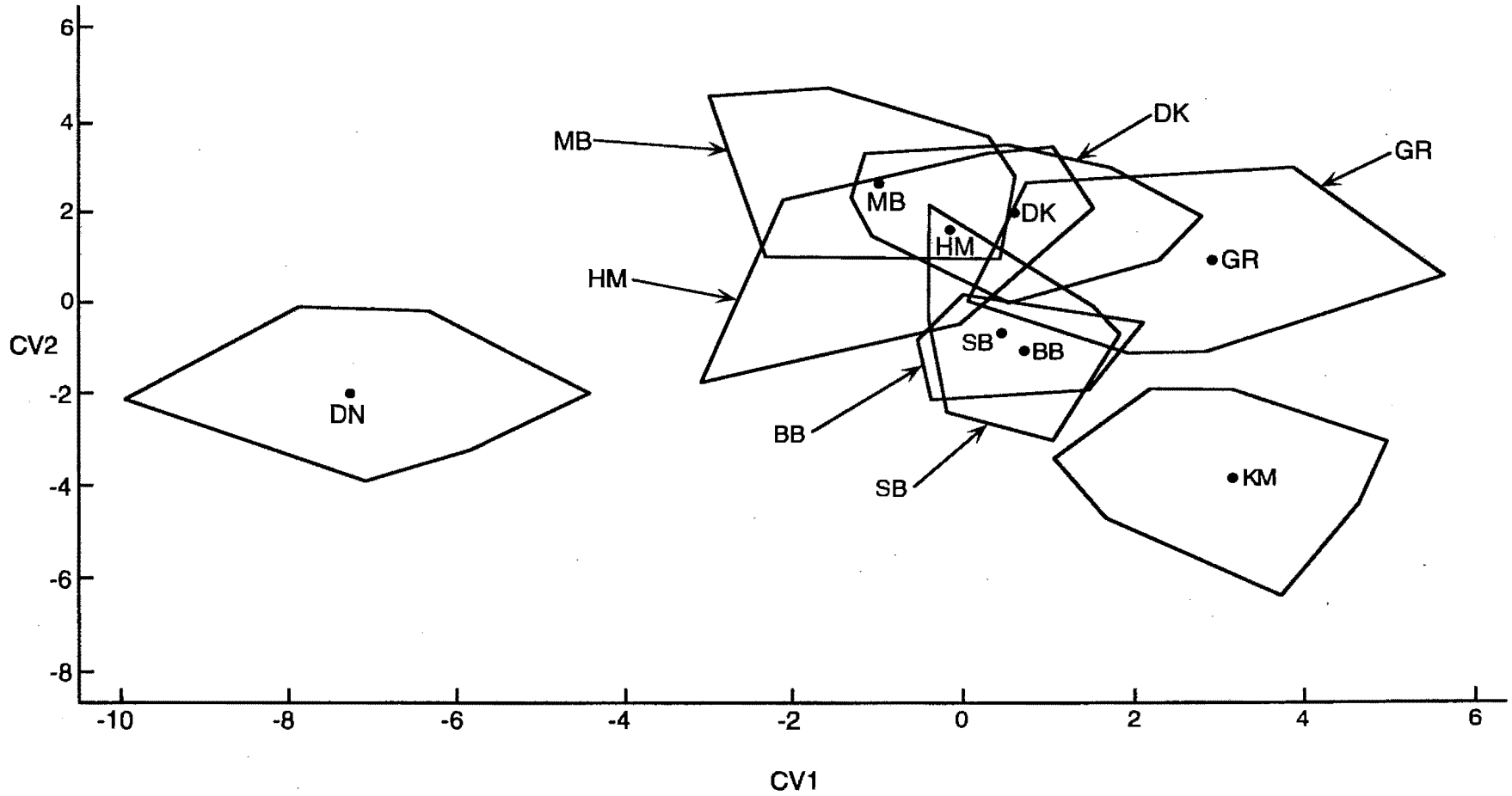


Figure 15. Plot of the first two canonincal variables for the populations of *B. lagenaria*. The outlines surround all of the points for each population. Abbreviations are as in Table 10.

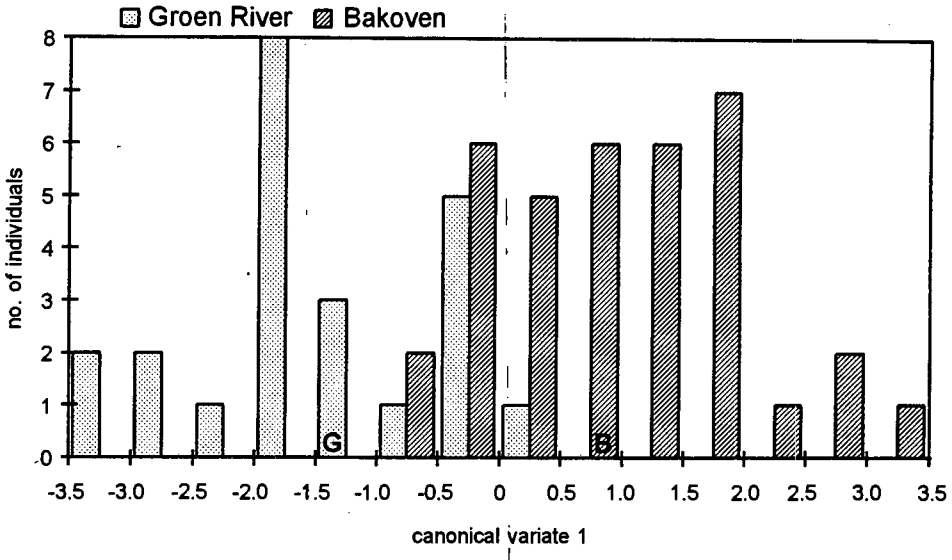


Fig. 16. Histogram of the first canonical variable for the two populations of *B.* sp. A. The position of the means (G=Groen River, B=Bakoven) are shown above the axis.

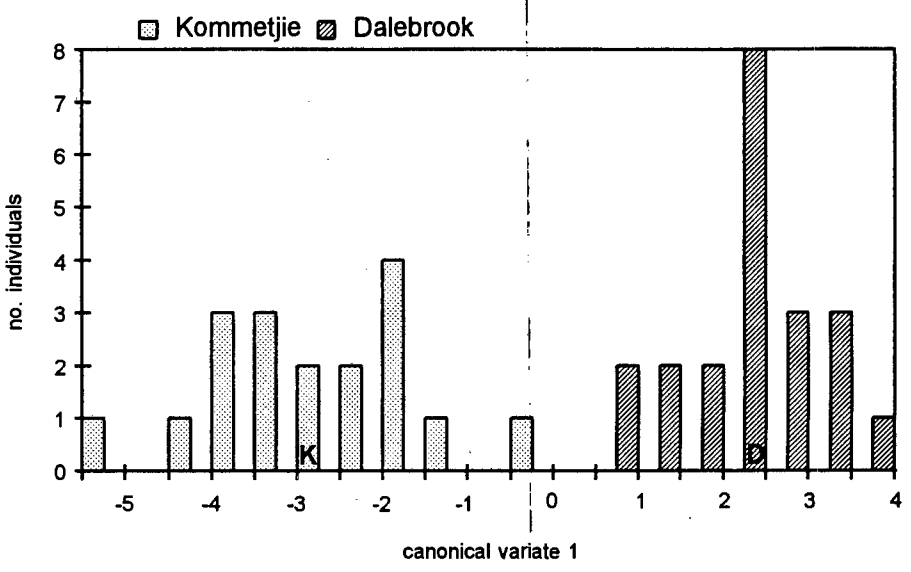


Fig. 17. Histogram of the first canonical variable for the two populations of *B. catarrhacta*. The position of the means (K=Kommetjie, D=Dalebrook) are shown above the axis.

height was useful in three of the species, the remaining variables with large contributions occurring in only two or fewer of the species. Of the 12 quantitative variables, only aperture width did not contribute much to the separation between the populations in any of the species, but three of the six qualitative variables (constriction, aperture sculpture and parietal scar) contributed little to the separation between populations. In all of the species, the plots of the first two canonical variates showed at least some separation between some of the populations.

Principal component analyses

The results of the principal components analyses showed similar trends for all of the species. The original 12 variables (11 and 8 for *B. cincta* and *B. pubescens* respectively) were reduced to a small number of components, the first three of which accounted for between 93% and 98% of the total variation (Table 17). As with the principal components analysis performed on all of the species together (Table 6), the first principal component in each of the species mainly represents variation due to size, since the elements of the first eigenvector are positive, are approximately of equal size (except for T1 and T2) and are very close to the predicted value of (number of characters)$^{-1/2}$ which equals 0.29 (0.30 and 0.35 for *B. cincta* and *B. pubescens* respectively). In all of the species, most of the variation of the second component was due to the thickness of the shell at the aperture (Table 17), whilst the elements that represented most of the variation in the third component were either shell thickness (*B. cincta* and *B. pubescens*), spire height (*B. papyracea* and *B. lagenaria*), or a combination of both (*B.* sp. A and *B. catarrhacta* - Table 17).

The proportion of the total variance accounted for by the first component was similar for most species, ranging from 80% to 89%, except for *B. lagenaria*, where this component only accounted for about 74% of the total variance (Table 17). However, the proportion accounted for by the second components was higher for this species, so that the overall proportion of the first three components was similar in all species.

Table 17. Principal component coefficients, eigenvalues and cummulative proportion of the total variance for each of the species of *Burnupena*

	Species																	
	B. papyracea			*B. c. cincta* & *B. c. limbosa*			*B. pubescens*			*B. lagenaria*			*B.* sp. A			*B. catarrhacta*		
Component	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Eigenvalue	10.70	0.83	0.19	8.78	1.06	0.43	6.50	1.00	0.26	8.92	1.85	0.62	10.20	0.90	0.35	10.16	1.02	0.31
Cumulative %	89.2	96.1	97.6	79.8	89.5	93.4	81.2	93.7	96.9	74.3	89.7	94.9	85.0	92.5	95.4	84.7	93.2	95.8
Eigenvectors:																		
variable																		
shell length	0.298	-0.158	0.354	0.328	-0.146	-0.093	0.380	-0.215	0.034	0.309	-0.204	0.319	0.304	-0.025	-0.346	0.307	0.011	-0.302
shell width	0.302	-0.081	-0.224	0.331	-0.037	-0.085	0.385	-0.104	-0.047	0.330	-0.002	-0.099	0.306	-0.149	0.058	0.307	-0.130	0.111
spire height	0.287	-0.163	**0.681**	0.310	-0.186	-0.203	0.362	-0.264	0.089	0.253	-0.263	**0.678**	0.267	0.270	**-0.765**	0.288	0.141	**-0.611**
aperture length	0.299	-0.108	-0.312	0.318	-0.118	-0.002	0.382	-0.133	-0.067	0.324	-0.083	-0.182	0.284	-0.373	0.200	0.304	-0.148	0.106
aperture width	0.300	-0.039	-0.332	0.327	-0.035	-0.019	0.380	-0.109	-0.064	0.318	0.025	-0.306	0.293	-0.295	0.031	0.305	-0.058	0.177
shoulder height	0.303	-0.085	0.000				0.382	-0.084	0.016	0.323	-0.078	0.007	0.299	-0.199	-0.051	0.295	-0.237	0.218
shell thickness 1	0.222	**0.698**	-0.150	0.206	**0.638**	**0.605**	0.268	**0.633**	**0.723**	0.120	**0.647**	0.220	0.260	**0.495**	0.159	0.241	**0.546**	**0.510**
shell thickness 2	0.237	**0.620**	0.302	0.205	**0.671**	**-0.478**	0.261	**0.660**	**-0.677**	0.125	**0.655**	0.116	0.243	**0.568**	**0.405**	0.225	**0.652**	-0.242
body weight	0.302	-0.122	0.003	0.321	-0.184	0.059				0.315	-0.116	-0.169	0.306	-0.144	0.036	0.297	-0.209	-0.256
shell weight	0.302	0.003	-0.079	0.315	0.092	-0.360				0.314	0.077	0.281	0.302	0.187	-0.012	0.305	0.101	0.200
operculum length	0.299	-0.152	-0.001	0.315	-0.102	0.235				0.312	-0.003	-0.246	0.290	-0.113	0.097	0.278	-0.303	0.007
operculum width	0.299	-0.109	-0.184	0.306	-0.133	0.400				0.310	0.091	-0.269	0.302	-0.045	0.219	0.299	-0.113	0.094

Values in bold indicate the variables that contribute the most to the variation in each component.

Plots of the principal components (not presented) showed either (a): a similar pattern of separation between the populations as that obtained using the canonical variables (*B. papyracea*, *B. pubescens*, *B.* sp. A and *B. catarrhacta*) although not as complete, or (b): essentially no separation between the populations along any of the axes (*B. cincta* and *B. lagenaria*). In the former case, the implication is that the group information used by the canonical analysis did not improve the separation of the populations, and that therefore the amount of variation within the populations is less than, or does not overshadow, the differentiation between the populations. In the case for *B. cincta* and *B. lagenaria*, the inability of the principal component analysis to differentiate between the populations (although the Durban population of *B. lagenaria* was partially separated), implies that much of the variability within these species is contained within the populations.

COMPARISONS BETWEEN SPECIES PAIRS OR GROUPS

In this final section of the results, discriminant and canonical variate analyses were performed on certain pairs of species. Only species which are not readily distinguishable have been compared. In this way, the analyses can be used to look for differences which discriminate specifically between the species of interest without being confounded by differences between other species. As was discussed in the introduction, there is little problem distinguishing certain species from each other, but for some species pairs, snail identification is difficult. Historically, the literature (see references in introduction) has many examples of so called 'intermediates' between certain species, and problems of assigning specimens to a species. The results in the first section of this study have indicated why this situation has arisen. The canonical analysis of all of the species showed that most of the species were morphometrically very similar to at least one other species, as evidenced by the amount of overlap between them (see Fig. 6a). Only *B.* sp. B was well separated from all of the other species, but this was due to one variable, the number of ribs. With this variable excluded, this species was indistinguishable from *B. papyracea* (Fig 6b).

Some of the pairs of species that have previously been confused, are not considered in this section, because the analyses in the first section of the results (in which all species were considered simultaneously), showed that these species pairs are readily separable. Specifically, both the discriminant and canonical analyses (see Tables 5a and 5c, Figs 6a and 6b) showed no difficulty in distinguishing between the following pairs of taxa:

- *B. cincta cincta* and *B. catarrhacta*
- *B. cincta limbosa* and *B. catarrhacta*
- *B. cincta cincta* and *B. pubescens*

The pairs that are considered below are as follows:

- *B. papyracea, B. pubescens* and *B.* sp. B (all from the Western Overlap only)
- *B. cincta* and *B. lagenaria*
- *B. cincta limbosa* and *B.* sp. A
- *B. lagenaria* and *B.* sp. A (from the West Coast only)
- *B. lagenaria* and *B. catarrhacta*
- *B. papyracea, B.* sp. A and *B. cincta limbosa* (from the West Coast only)

This section also examines the group placement of a number of specimens which could not be assigned to a species, as well as some of the type specimens.

***B. papyracea, B. pubescens* and *B. sp. B* from the Western Overlap**

The shells of these three of the species were covered by a bryozoan which had to be removed before the snail could be identified. For some of the animals, however, identification based on morphometrics was not clear-cut and errors were made which were later detected by allozyme electrophoresis (see Chapter 3). The confusion arose between snails collected at A-Frame (AF) and Castle Rock (CR) in the Western Overlap region, where all three species occurred together. Neither *B. pubescens* nor *B.* sp. B was found on the West Coast where the other populations of *B.*

papyracea were sampled. A discriminant analysis using nine of the 18 variables was applied to these two sites (Table 18b). The jackknifed classification of the five populations, PAP-AF, PAP-CR, PUB-AF, PUB-CR AND B-AF, indicated that almost 90% of the snails could be identified to the correct population (Table 18a), although only for the PAP-AF population were all of the individuals correctly identified. There was one *B. papyracea* individual that was allocated to *B. pubescens*, and two each from *B. pubescens* and *B.* sp. B were allocated to *B. papyracea*.

A plot of the first two canonical variates (Fig. 18a) showed a complete separation of *B.* sp. B from the other two species. There was a slight overlap of PAP-AF with PAP-CR, as was the case when this species was analysed separately (Fig. 12). There was partial to complete overlap of the two *B. pubescens* populations and the PAP-CR population. The PAP-CR population displayed greater overlap with the two *B. pubescens* populations than it did with PAP-AF. This result was most probably due to the smaller mean size of animals in the PAP-CR population, which was similar to those of the *B. pubescens* populations (Fig. 9). The mean size of the B-AF population was intermediate between the two *B. papyracea* populations (Fig. 9), which would account for its placement between these two along the first axis (Fig. 18a). The first canonical variate in this analysis clearly had a large size component. The standardized coefficients of the canonical variables (Table 18b) show this to be the case: shell length had a large positive coefficient for the first variable. In terms of shape, relative shell thickness and weight (Appendix A), there were only small differences between these five populations although, as was noted previously, the PAP-AF population was slightly narrower (Fig. 10a), with a longer spire (Fig. 10b), and the PAP-AF and B-AF populations appeared to have slightly heavier body and shell weights (Fig. 10d).

Apart from *B.* sp. B, there was no separation between the populations along the second axis (Fig. 18a) which was influenced mainly by shell length and spire height (Table 18b). There was however, some separation between the populations along the axis of the third canonical variable (Fig. 18b) which was achieved mainly by the contrast of shell length and weight with opercular width and shoulder height (Table 18b). This latter canonical variable accounted for almost as much variation within the original variables as did the second canonical variable (17% and 20%

Table 18. Discriminant and canonical variate analyses of the A-Frame and Castle Rock populations of *B. papyracea, B. pubescens* and *B.* sp. B. (a) Jackknifed classification, showing the percentage of correctly classified snails for each population, and the number of cases classified into each species. (b) the standardized coefficients for the canonical variables.

(a) Jackknifed classification

		no. of cases classified into species			
population	%correct	PAP	PUB	B	n
PAP-CR	90.0	39	**1**	0	40
PAP-AF	100.0	30	0	0	30
PUB-CR	80.0	**2**	28	0	30
PUB-AF	85.0	0	20	0	20
B-AF	86.7	**2**	0	13	15
total	88.9	73	49	13	135

The values in bold indicate the numbers of animals placed into the wrong species.

(b) Standardized coefficients

	canonical variables		
	1	2	3
variable			
shell length	**1.52**	**-4.19**	**1.26**
spire height	**1.31**	**3.96**	0.33
shoulder height	-0.51	**2.20**	**-1.01**
shell thickness 2	0.76	0.24	0.05
shell weight	**-3.00**	-1.47	**1.23**
opercular length	0.76	-0.56	-0.22
opercular width	0.09	-0.61	**-1.50**
constriction	-0.26	0.07	0.60
spire angle	0.23	0.90	0.02
cumulative % of total variance	57.2	77.5 (20.3%)	94.9 (17.4%)

The values in bold indicate the variables that contribute most to the separation of the groups.

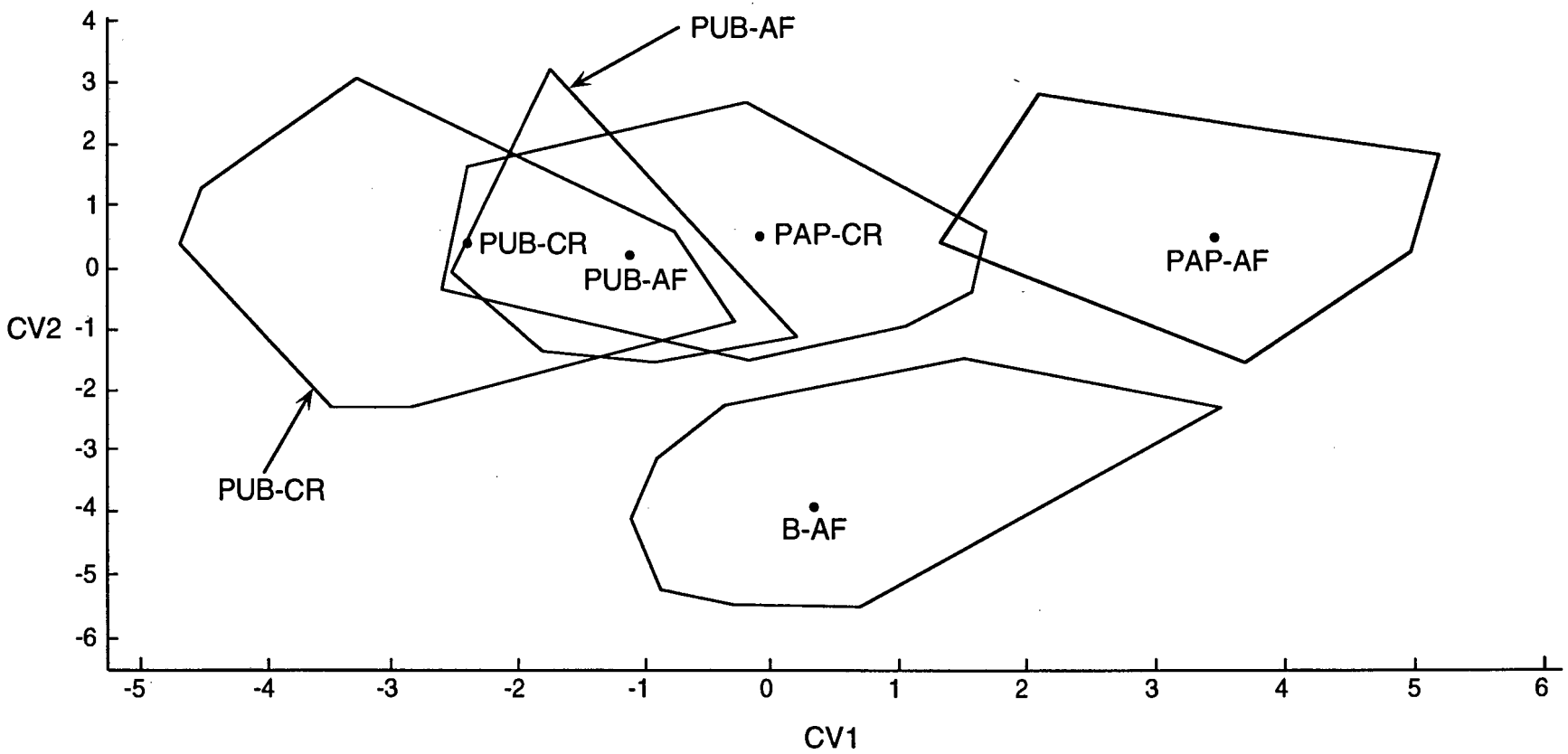


Figure 18a. Plot of the first two canonical variables for the A-Frame and Castle Rock populations of *B. papyracea*, *B. pubescens* and *B.* sp. B.

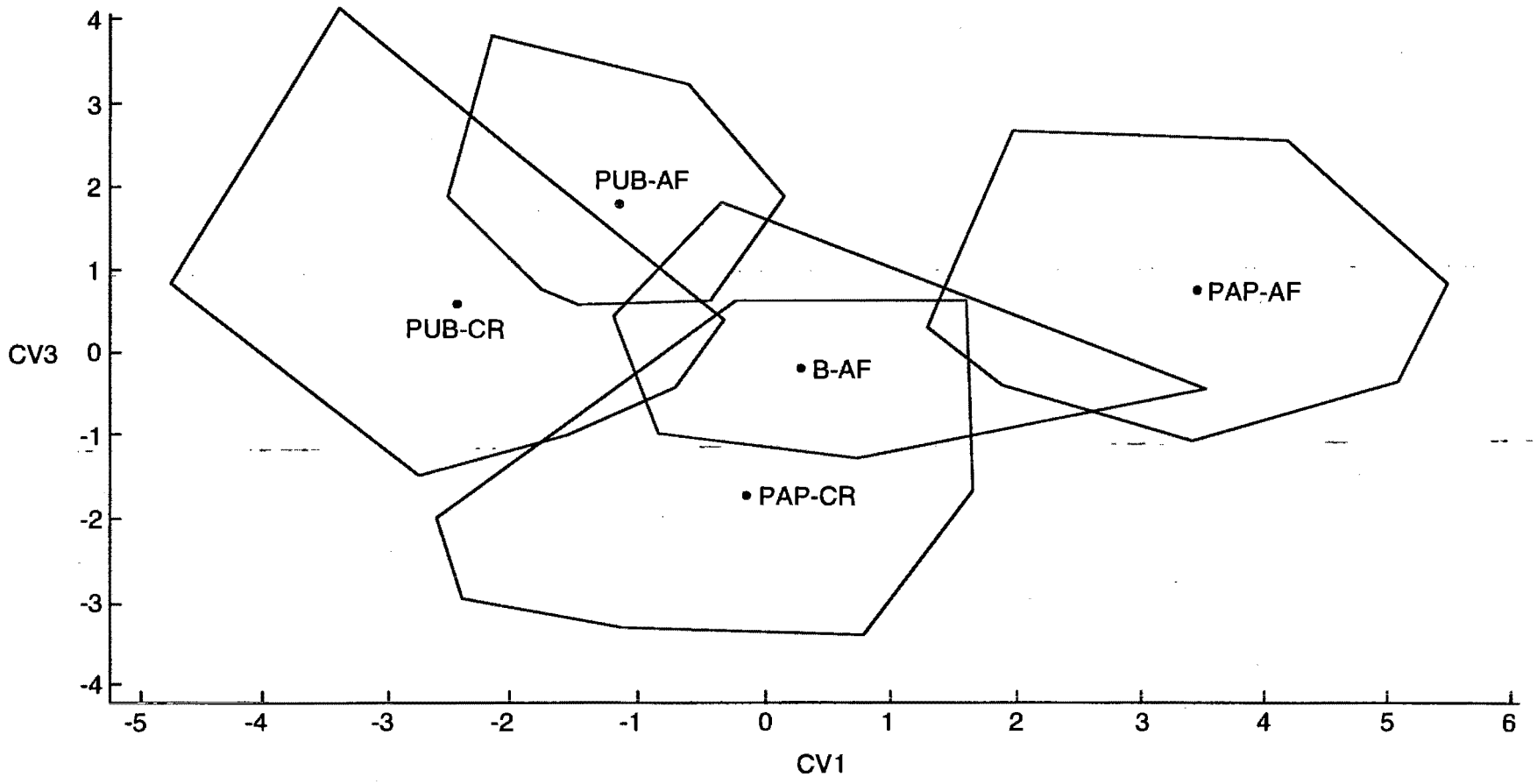


Figure 18b. Plot of the first and third canonical variables for the A-Frame and Castle Rock populations of *B. papyracea*, *B. pubescens* and *B.* sp. B.

respectively - Table 18b). Here the PAP-CR population showed only slight overlap with the *B. pubescens* populations. Between the three canonical axes, these three species can be more or less separated from each other.

The two rib variables, strength and number, could not be used to aid discrimination between these populations, since they were invariant in each of the populations (Fig. 11a-b). However, although the *B. papyracea* and *B. pubescens* were identical for these variables in the Western Overlap, the *B.* sp. B snails had weaker (Fig. 11a) and more numerous ribs (Fig. 11b), and these variables could therefore be used to separate these animals from *B. papyracea* and *B. pubescens*. One of the distinctive features of *B. pubescens* shells is the presence of fine axial ridges which together with the spiral ribs, give the shell a cancellate granular texture which is most noticeable on the spire. This feature can be used to differentiate between the shells of *B. papyracea* and *B. pubescens* most of the time. Occasionally the axial ridges are weak or absent, and it is with these animals that problems of identification arise. The new species, *B.* sp. B, whilst having more, and weaker ribs than *B. pubescens*, was also found to have a cancellate spire, although this is not as pronounced as in typical *B. pubescens*.

A second discriminant analysis was done on the *B. papyracea* - *B. pubescens* - *B.* sp. B complex, but this time it included the *B. pubescens* population from Rooiels (PUB-RE). It also included a number of cases (described below) which although not used in the calculation of the discriminant function, were classified by the function. The latter included a number of animals collected in the Western Overlap, which belonged to the *B. papyracea* - *B. pubescens* - *B.* sp. B complex by virtue of their being covered by the bryozoan, but were difficult to identify. Three animals from Castle Rock were initially assigned to *B. papyracea*, but later shown to belong to *B. pubescens* (see Chapter 3). A further three animals were collected which belonged to *B.* sp. B. These animals were not included with the B-AF population since two of them were collected at Rooiels and one at Castle Rock. All three were smaller than the animals collected at A-Frame, and, as has already been established, there are differences between populations from different sites. The Castle Rock animal was initially identified as *B. pubescens*, but was reassigned to *B.* sp. B after the

electrophoretic analysis. The sample collected from Rooiels contained a number of animals which clearly belonged to *B. pubescens*, two which were clearly *B.* sp. B and a further six that could not be assigned to a species. These six snails appeared to look more similar to *B. pubescens* in terms of their overall shape and shell colour, although their ribbing was more like *B.* sp. B. The results of the electrophoresis for these snails were inconclusive (see Chapter 3). These latter six animals, plus the three Castle Rock *B. pubescens* and the three extra *B.* sp. B, were entered as new cases into the second discriminant analysis.

The results of these analyses are shown in Table 19 and Fig. 19. The inclusion of the *B. pubescens* population from Rooiels had an effect on the number of correctly identified snails, reducing the overall percentage from 89% to 81% (Table 19a). Not all of the same variables were used in this analysis (Table 19b), although the overall pattern of the plot of the first two canonical variables remained essentially the same, there being slightly more overlap between the populations (Fig. 19 compared with Fig. 18a). The results of the classification of the 12 new cases are given in Table 19a. Of the three Castle Rock *B. pubescens* individuals that were initially misidentified as *B. papyracea*, one was identified as being most similar to the PUB-CR population, but the other two were identified as being more similar to the PAP-CR population. The *B.* sp. B individual collected from Castle Rock was identified as being most similar to the B-AF population, but the two *B.* sp. B individuals from Rooiels were allocated to the PUB-RE population. Of the six snails from Rooiels which could not be identified, three were allocated to PUB-CR, one was identified as being most similar to the PUB-AF population, and two were placed with B-AF. None were allocated to the PUB-RE population! The positions of these 12 snails relative to the other populations are shown in Fig. 19.

As noted above, the rib variables were not used since they were invariant in each of the populations, but both could be used to distinguish the new species, *B.* sp. B, from *B. papyracea* and *B. pubescens*. If these variables had been used, then the six unidentified Rooiels snails would probably have been allocated to *B.* sp. B. However, four of the six were found to be more similar to

Table 19. Discriminant and canonical variate analyses of the A-Frame, Castle Rock and Rooiels populations of *B. papyracea, B. pubescens* and *B*. sp. B. The discriminant functions were used to identify 12 new cases. (a) Jackknifed classification, showing the percentage of correctly classified snails for each population, and the allocation of the new cases. (b) the standardized coefficients for the canonical variables.

(a) Jackknifed classification

		allocation of new cases		
population	%correct	PUB-CR?[1]	B-CR/RE[2]	RE ?[3]
PAP-CR	78.0	2	-	-
PAP-AF	90.0	-	-	-
PUB-CR	80.0	1	-	3
PUB-AF	65.0	-	-	1
PUB-RE	100.0	-	2	-
B-AF	87.5	-	1	2
total	80.9	3	3	6

[1] snails collected at CR and initially identified as *B. papyracea*

[2] *B*. sp. B snails collected at CR and RE

[3] snails collected at RE. Identification uncertain

(b) Standardized coefficients

	canonical variables	
	1	2
variable		
shell width	-0.70	**-1.81**
spire height	**1.76**	0.61
aperture length	0.68	**-2.05**
shoulder height	**-0.91**	**2.75**
shell thickness 2	0.39	0.33
constriction	-0.38	-0.22
aperture sculpture	-0.19	-0.41
parietal scar	0.18	0.08
spire angle	0.10	0.67
cumulative % of total variance	44.7	74.3

The values in bold indicate the variables that contribute most to the separation of the groups.

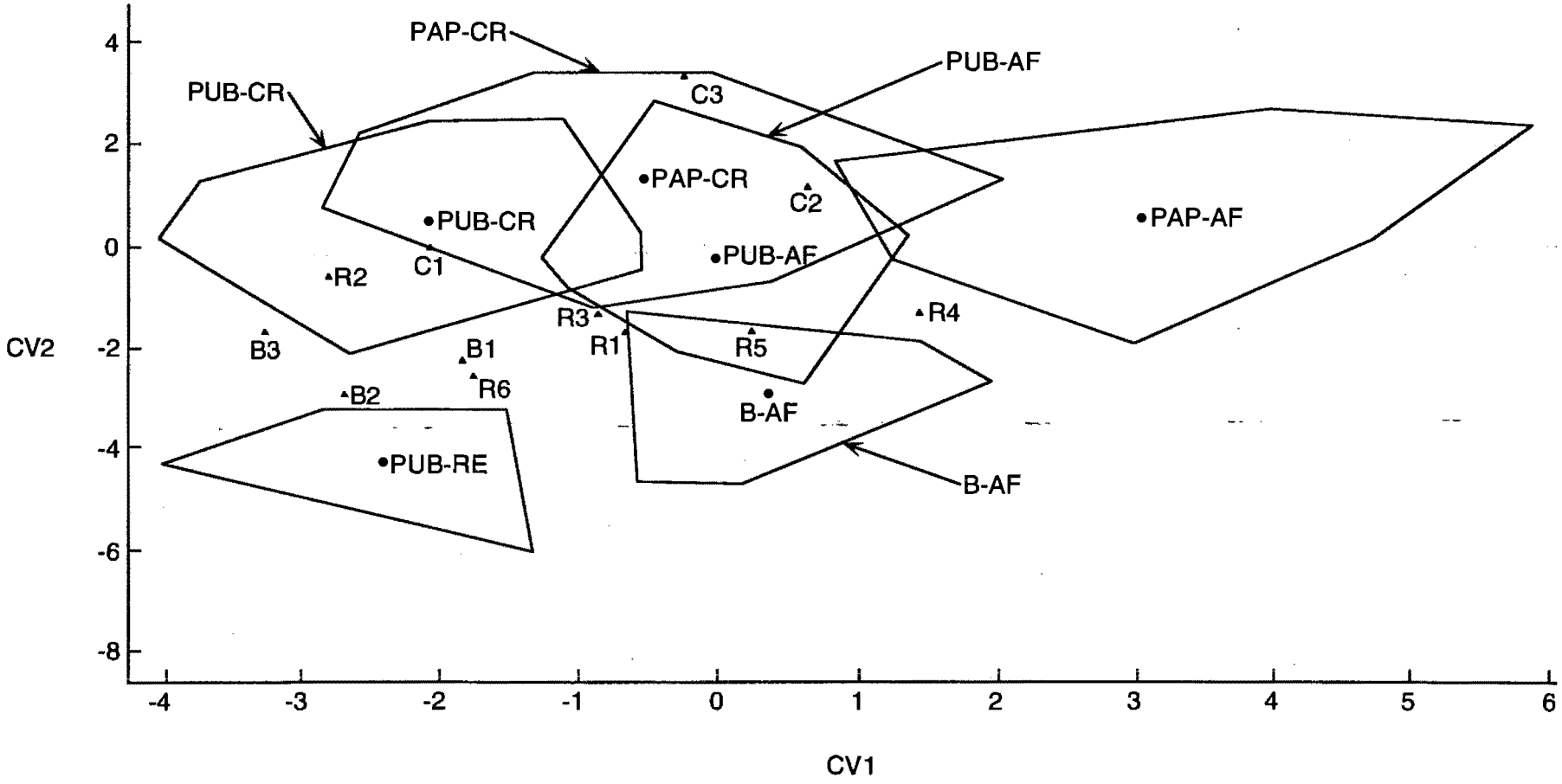


Figure 19. Plot of the first two canonical variables for the A-Frame, Castle Rock and Rooiels populations of *B. papyracea*, *B. pubescens* and *B.* sp. B. The positions of the new cases (▲) identified by the discriminant function (see Table 19) are indicated, with the following abbreviations: C1-C3 = snails collected at Castle Rock; B1-B3 = *B.* sp. B snails collected at Castle Rock and Rooiels; R1-R6 = snails collected at Rooiels. The abbreviations for the centroids (•) are as in Table 1.

B. pubescens as noted. Both of the *B.* sp. B from Rooiels were also found to be more similar to *B. pubescens.*

In summary: of the misallocated individuals in the first analysis of the AF and CR populations, ten were allocated to the correct species but the wrong locality and five were allocated to the wrong species, four of these to the correct site. Of the six new cases where the species identification was known, only one was allocated to the correct species and locality, one was allocated to the correct species but the wrong site, and the remaining four were allocated to the wrong species but correct site. The other six cases where species identification was not unambiguously established, none were allocated to the same locality, with four found to be most similar to *B. pubescens* and two to *B.* sp. B. The two rib variables could not be used, but they indicated that these latter six individuals were most probably *B.* sp. B.

B. cincta* and *B. lagenaria

Both of these species have large geographic ranges that overlap considerably. In the previous section, where each species was considered separately, both species exhibited a substantial overlap between most of the populations examined (see Fig. 13 for *B. cincta* and Fig. 15 for *B. lagenaria*). A canonical variate analysis of all eight populations from each species showed essentially the same results: for *B. cincta*, the three West Coast *B. cincta limbosa* populations were separated from the Western Overlap and South Coast *B. cincta cincta* populations, which overlapped substantially, and for *B. lagenaria*, LAG-DN and LAG-KM were separated from the other populations, amongst which there was little separation. Overlap between the species was however, limited to only a few populations. Similar results were obtained by using regions as the grouping variable. The latter approach was used to keep the plot less cluttered. The results of this analysis are given in Table 20. Almost 95% of the individuals were assigned to the correct group (Table 20a). As expected, all of the LAG-DN (East Coast) individuals were correctly placed. About 2% of the *B. cincta* individuals (from the West Coast and Western Overlap) were identified as being more similar

Table 20. Discriminant and canonical variate analyses of *B. cincta* (CIN & LIM) and *B. lagenaria* (LAG), grouped by region. (a) Jackknifed classification, showing the percentage of correctly classified snails for each population, and the number of cases classified into each species. (b) the standardized coefficients for the canonical variables.

(a) Jackknifed classification

region[1]	%correct	cases classified into species CIN/LIM	LAG	n
LIM-WC	93.1	54	**4**	58
CIN-WO	99.2	120	**1**	121
CIN-SC	88.7	62	0	62
LAG-WC	92.0	**3**	72	75
LAG-WO	92.2	0	64	64
LAG-SC	96.9	0	32	32
LAG-EC	100.0	0	25	25
total	94.5	239	198	437

The values in bold indicate the numbers of animals placed into the wrong species.
[1] WC = West Coast; WO = Western Overlap; SC = South Coast; EC = East Coast

(b) Standardized coefficients

	canonical variables 1	2
variable		
body width	0.15	**-1.71**
spire height	**0.87**	-0.40
aperture length	0.15	-0.19
aperture width	**-0.70**	**0.80**
shoulder height	-0.49	0.36
shell thickness 1	-0.07	0.19
body weight	**-0.79**	**-1.27**
shell weight	0.41	0.34
opercular length	0.23	0.14
opercular width	**0.75**	**1.24**
rib strength	**1.09**	0.25
no. ribs	-0.59	0.02
constriction	-0.01	0.49
aperture sculpture	-0.25	-0.24
parietal scar	0.03	-0.23
spire angle	0.02	0.19
cumulative % of total variance	64.2	81.4

The values in bold indicate the variables that contribute most to the separation of the groups.

to West and South Coast *B. lagenaria*. None were considered more similar to the Western Overlap *B. lagenaria*. Three *B. lagenaria* individuals (from the West Coast) were identified as being more similar to *B.* c. *limbosa*. The only variables not used in the classification function were shell length and shell thickness 2 (Table 20b).

A plot of the first two canonical variates (which accounted for about 81% of the total variation) is shown in Fig. 20. The variables that contributed the most towards the separation of the groups are shown in Table 20b. As expected, the LAG-DN population was well separated from the other groups. Within each species, the pattern of regional overlap followed that described above. Between species, most of the overlap was within the West Coast region. *B.* c. *limbosa* in this region also overlapped partially with *B. lagenaria* from the Western Overlap. There was only slight overlap of the South Coast *B.* c. *cincta* with both South Coast and Western Overlap *B. lagenaria*, and there was no overlap between the two species in the Western Overlap.

The discriminant analysis was repeated using the three taxa above, namely *B.* c. *cincta*, *B.* c. *limbosa* and *B. lagenaria*. A number of additional individuals were included which were not used in the calculation of the discriminant function. These were entered as new cases into the discriminant analysis and classified according to the function, thereby allowing assessment of which species they most closely resemble morphometrically. These new cases included Lamarck's type specimens of *B. lagenaria* (3 syntypes) and *B. limbosa* (2 syntypes), and Quoy and Gaimard's (1832) *Buccinum violaceum* type specimen. *B.* sp. A and *B. catarrhacta* were also included in the calculations of the discriminant functions, since they both showed some overlap with the above taxa when all species were analysed together.

In terms of individuals allocated to the wrong species, the discriminant analysis showed similar results to those obtained when all species were analysed together (Table 21). Of interest here was the placement of the type specimens based on the calculations for the five species (*B.* c. *cincta*, *B.* c. *limbosa*, *B. lagenaria*, *B.* sp. A and *B. catarrhacta*). The two *B. limbosa* syntypes were found to be most similar to *B. cincta limbosa* (with posterior probabilities of 0.78 and 0.87), and all

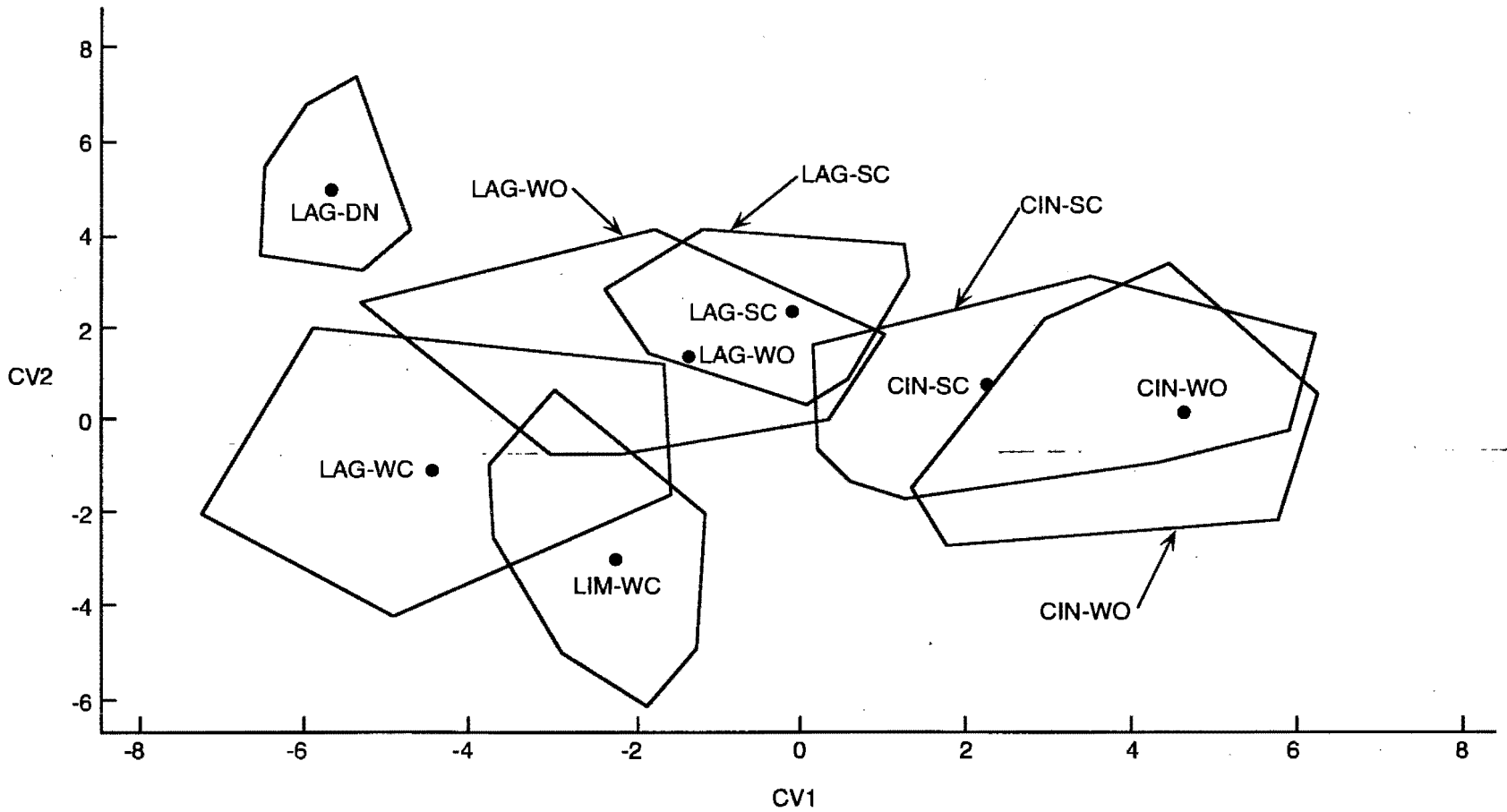


Figure 20. Plot of the first two canonical variables for the populations of *B. cincta* and *B. lagenaria*, grouped by region. Outlines surround 100% of the points for each group. See Table 20 for abbreviations.

Table 21. Discriminant and canonical variate analyses for the species *B. cincta cincta*, *B. cincta limbosa*, *B. lagenaria*, *B.* sp. A and *B. catarrhacta*. The discriminant functions were used to identify the following type specimens: *B. limbosa* (Lamarck), *B. lagenaria* (Lamarck) and *Buccinum violaceum* (Quoy and Gaimard). (a) Jackknifed classification, showing the percentage of correctly classified snails for each species, and the allocation of the types. (b) the standardized coefficients for the canonical variables.

(a) Jackknifed classification

		no. of cases classified into species					
species	%correct	CIN	LIM	A	LAG	CAT	n
CIN	99.5	189	0	0	**1**	0	190
LIM	82.8	0	48	**9**	**1**	0	58
A	100.0	0	0	59	0	0	59
LAG	90.3	**2**	**6**	**9**	186	**3**	206
CAT	97.5	0	0	0	**1**	39	40
B. limbosa	-	0	**2**	0	0	0	2
B. lagenaria	-	0	0	0	**3**	0	3
B. violaceum	-	0	0	**1**	0	0	1
total	94.2	191	56	78	192	42	559

The values in bold indicate the numbers of animals placed into the wrong species, as well as the allocation of the type specimens.

(b) Standardized coefficients

	canonical variables	
	1	2
variable		
shell width	-0.09	**-0.96**
spire height	-0.42	-0.39
aperture length	**1.12**	-0.47
aperture width	-0.34	**1.36**
shell thickness 1	-0.02	0.19
shell thickness 2	0.06	-0.22
rib strength	**0.86**	-0.10
no. ribs	0.05	0.51
constriction	0.09	0.51
aperture sculpture	-0.05	-0.14
parietal scar	-0.34	0.25
spire angle	-0.19	0.12
cumulative % of total variance	60.4	84.7

The values in bold indicate the variables that contribute most to the separation of the groups.

three *B. lagenaria* type specimens were allocated to *B. lagenaria* (posterior probabilities 0.99, 1.0 and 1.0). *Buccinum violaceum* was found to be most similar to *B.* sp. A, but the posterior probability was only 0.426. The posterior probability for allocating this specimen to *B. catarrhacta* was 0.417 and the specimen is very worn. Thus, although *B. violaceum* and *B.* sp. A may belong to the same species, this interpretation must be regarded with caution.

***B. cincta limbosa* and *B.* sp. A**

As noted above, *B.* sp. A has only been found on the West Coast, and has been confused with populations of *B. c. limbosa* collected in this region, mainly because the latter does not show the characteristic ribbing found on the shells of the *B. c. cincta* animals from the Western Overlap and South Coast regions (Fig. 11a). Although *B.* sp. A has not been described, it is fairly common, and many of the specimens of it located in the South African and British Museums have been misidentified as *B. limbosa* (personal observation). When all of the species were considered together, there was substantial overlap between these two species (Fig. 6a), and, although all of the *B.* sp. A snails were correctly identified by the discriminant analysis, all of the *B. c. limbosa* snails that were incorrectly identified were allocated to *B.* sp. A (Table 5a). When *B. c. limbosa* and *B.* sp. A were analysed in isolation by discriminant analysis, the jackknifed classification showed that almost 93% of the animals were allocated to the correct population, and that no *B. c. limbosa* were identified as *B.* sp. A , or vice versa (Table 22a).

Ten of the 18 variables (Table 22b) were used to calculate the classification functions. The first canonical variable accounted for about 75% of the total variation, whilst the second and third canonical variables accounted for 13% and 7% respectively (Table 22b). The variables that contributed most to the separation of the populations along each axis are indicated in Table 22b. A plot of the first two canonical variables showed almost complete separation between the three *B. c. limbosa* populations, partial overlap of the two *B.* sp. A populations, and partial overlap of the LIM-BB and A-BO populations. However, a plot of the first and third canonical variables (Fig. 21)

Table 22. Discriminant and canonical variate analyses of *B. cincta limbosa* and *B*. sp A. (a) Jackknifed classification, showing the percentage of correctly classified snails for each population, and the number of cases classified into each species. (b) the standardized coefficients for the canonical variables.

(a) Jackknifed classification

		cases classified into species		
population	%correct	LIM	A	n
LIM-BB	100.0	17	0	17
LIM-OK	96.3	27	0	27
LIM-KM	96.9	32	0	32
A-GR	82.6	0	23	23
A-BO	88.9	0	36	36
total	92.6	76	59	135

(b) Standardized coefficients

	canonical variables		
	1	2	3
variable			
shell length	**-1.79**	0.63	-0.06
shell width	**-1.28**	0.18	0.52
shell thickness 1	0.70	-0.18	0.46
shell thickness 2	0.13	**1.12**	-0.24
body weight	**3.13**	**-1.88**	**-2.55**
shell weight	**-1.17**	0.27	-0.68
opercular length	-0.75	0.04	0.97
opercular width	**1.19**	-0.07	**2.14**
constriction	-0.47	0.11	0.2
spire angle	0.38	0.60	-0.17
cumulative % of total variance	75.6	89.1 (13.5%)	96.3 (7.2%)

The values in bold indicate the variables that contribute most to the separation of the groups.

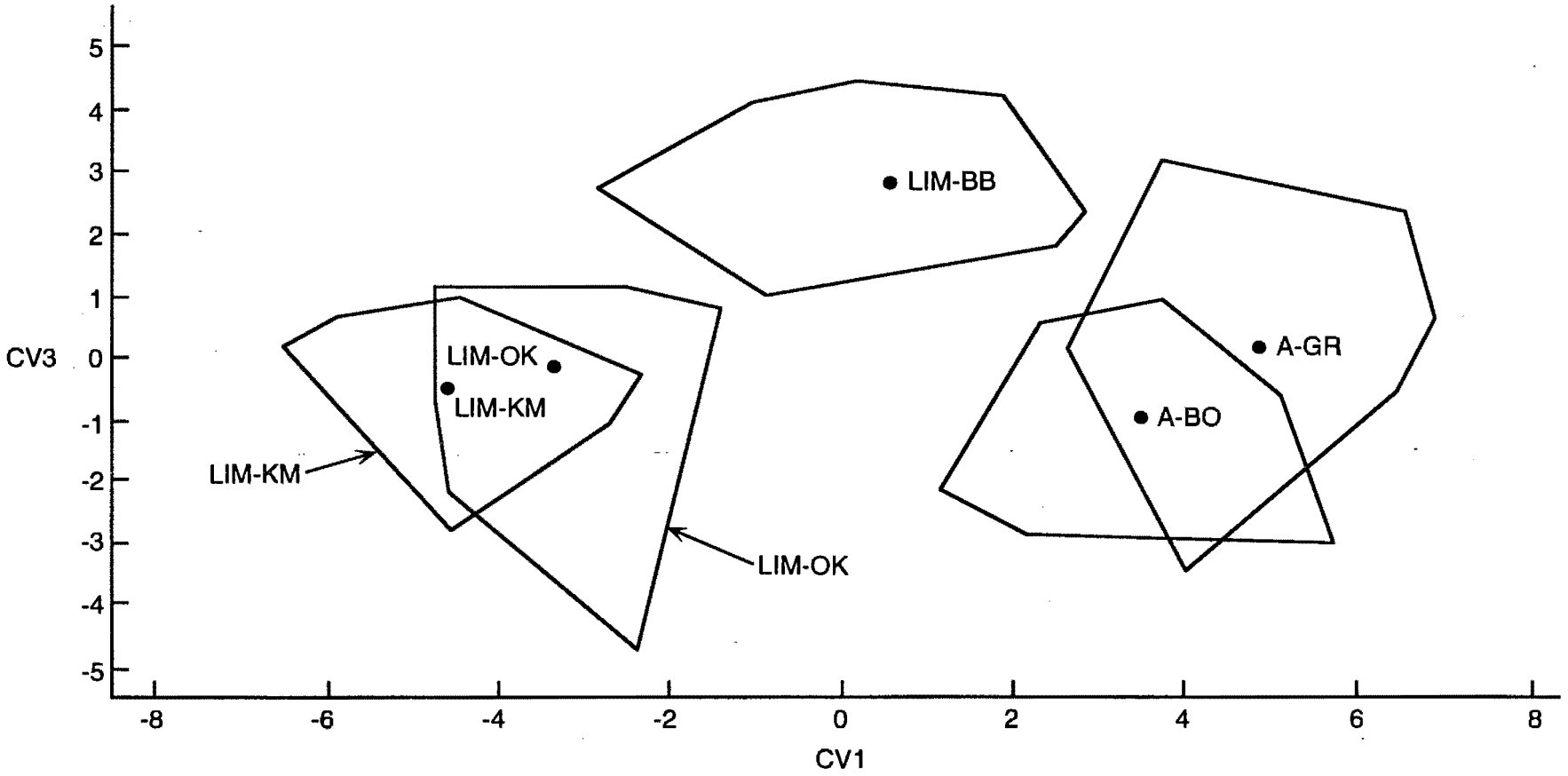


Figure 21. Plot of the first and third canonical variables for the populations of *B. cincta limbosa* and *B.* sp. A.

showed complete separation between the two species, although with greater within-species overlap. Most of the separation between the two species occurred along the first canonical variable. The LIM-KM and LIM-OK populations showed substantial overlap, as did the two *B.* sp. A populations. The LIM-BB population was separated from the other populations along the third axis, which mainly contrasted body weight and operculum width. This plot reflected more closely the results of the jackknifed classification (Table 22a).

***B. lagenaria* and *B.* sp. A**

These two species were found to have very similar shapes, being relatively wider, with a shorter spire and consequently longer aperture, and having a higher shoulder than most of the other species (Table 5 and Fig. 4). This was reflected in the plot of the canonical variables for all of the species (Fig. 6a) which showed partial overlap of these two species, and the discriminant analysis indicated that a few *B. lagenaria* snails were more similar to *B.* sp. A (Table 5a). When the qualitative variables were excluded, these two species overlapped almost completely (Fig. 6b), and relatively large number of each species were identified as being more similar to the other species (Table 5b). Hence, when all of the species were considered simultaneously, confusion between these two species occurred.

Discriminant and canonical variate analyses of all populations of these two species (i.e. eight *B. lagenaria* and two *B.* sp. A) showed that cases of misallocation and overlap between the species only occurred with the West Coast *B. lagenaria* populations. Since *B.* sp. A has only been found on the West Coast, the comparison between these two species was repeated using only the three West Coast *B. lagenaria* populations.

The jackknifed classification of the discriminant analysis found that almost 92% of the individuals were allocated to the correct population (Table 23a). Within species, a few individuals were allocated to the wrong population. Two individuals, both from Groen River, were allocated to

Table 23. Discriminant and canonical variate analyses of *B. lagenaria* and *B*. sp. A from the West coast. (a) Jackknifed classification, showing the percentage of correctly classified snails for each population, and the number of cases classified into each species. (b) the standardized coefficients for the canonical variables.

(a) Jackknifed classification

		cases classified into species		
population	%correct	LAG	A	n
LAG-GR	90.9	32	**1**	33
LAG-BB	92.9	14	0	14
LAG-KM	100.0	28	0	28
A-GR	87.0	**1**	22	23
A-BO	88.9	0	36	36
total	91.8	75	59	134

The values in bold indicate the numbers of animals placed into the wrong species.

(b) Standardized coefficients

	canonical variables	
	1	2
variable		
spire height	0.23	**-0.58**
aperture width	**-1.15**	-0.09
shell thickness 1	0.37	**0.60**
shell thickness 2	0.73	-0.14
body weight	**1.62**	0.31
shell weight	**-1.41**	-0.10
no. ribs	-0.16	**0.58**
constriction	-0.42	0.48
aperture sculpture	-0.19	-0.42
spire angle	**0.96**	0.28
cumulative % of total variance	68.3	92.3

The values in bold indicate the variables that contribute most to the separation of the groups.

the wrong species; one from LAG-GR was placed with A-BO and one from A-GR was identified as LAG-GR (Table 23a).

A plot of the first two canonical variates (containing 92% of total variance) is shown in Fig. 22, and the variables which contributed most to the separation between the populations are given in Table 23b. The overlap between the species was with both populations of *B.* sp. A and the LAG-GR population. *B.* sp. A was well separated from the other *B. lagenaria* populations.

B. lagenaria* and *B. catarrhacta

In the literature, most of the problems encountered in distinguishing these two species occurred in populations on the West Coast. Discriminant analyses using the populations as the *a priori* groups showed that overall, about 87% of the individuals were placed into the correct species and population (Table 24a), with most of the misclassifications placed into other populations of the correct species. Only three individuals were identified as being more similar to individuals of the other species and they all involved Kommetjie. Two LAG-KM individuals were allocated to the CAT-KM population, and one CAT-DK individual was allocated to the LAG-KM population. All but four of the variables (shell length and width, aperture width and shell thickness 1) were used in the classification function (Table 24b).

A plot of the first two canonical variates, which accounted for about 66% of the total variance, is shown in Fig. 23. For the sake of clarity, five *B. lagenaria* populations (BB, DK, SB, HM and MB) are grouped together on the plot although their respective means are shown. The variables that contributed the most to the separation of the populations are given in Table 24b. The within-species separations were consistent with those obtained in the earlier analyses (see Figs 15 and 17). *B. catarrhacta* was well separated from *B. lagenaria*, except for the Kommetjie population. The plot shows the partial overlap of the LAG-KM population with CAT-KM, and slight overlap with CAT-DK. The separation of *B. catarrhacta* and LAG-KM from the other *B. lagenaria* populations was mainly

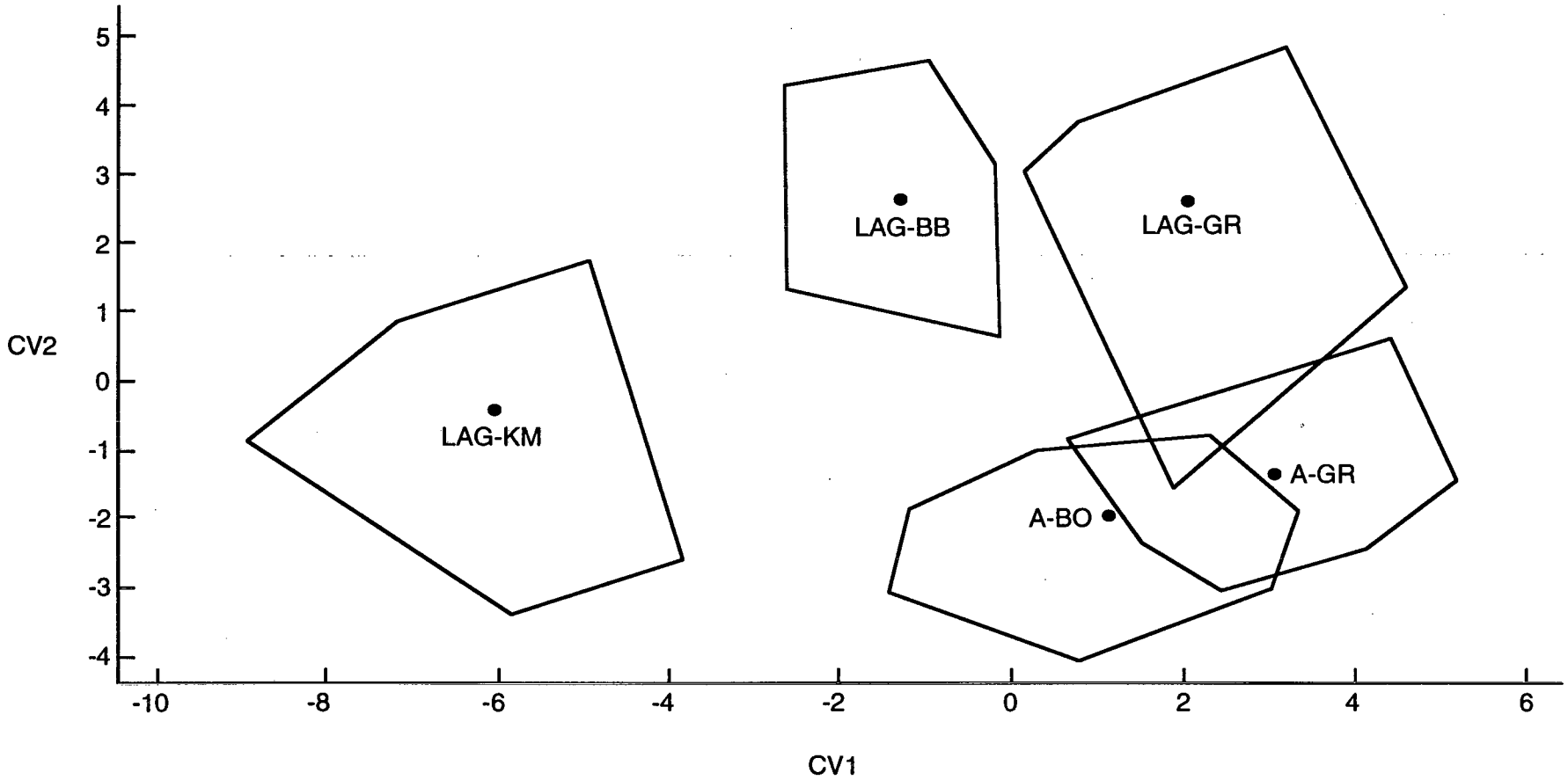


Figure 22. Plot of the first two canonical variables for the populations of *B.* sp. A and the West Coast *B. lagenaria* populations.

Table 24. Discriminant and canonical variate analyses of *B. lagenaria* and *B. catarrhacta*. (a) Jackknifed classification, showing the percentage of correctly classified snails for each population, and the number of cases classified into each species. (b) the standardized coefficients for the canonical variables.

(a) Jackknifed classification

		cases classified into species		
population	%correct	LAG	CAT	n
LAG-GR	81.8	33	0	33
LAG-BB	85.7	14	0	14
LAG-KM	92.9	26	**2**	28
LAG-DK	84.4	32	0	32
LAG-SB	93.3	15	0	15
LAG-HM	64.7	17	0	17
LAG-MB	90.6	32	0	32
LAG-DN	100.0	25	0	25
CAT-KM	83.3	0	18	18
CAT-DK	90.5	**1**	20	21
total	87.2	195	40	235

The values in bold indicate the numbers of animals placed into the wrong species.

(b) Standardized coefficients

	canonical variables	
	1	2
variable		
spire height	**-0.86**	0.61
aperture length	-0.44	**0.78**
shoulder height	-0.01	**-0.77**
shell thickness 2	0.17	0.36
body weight	**-1.12**	**0.75**
shell weight	0.30	-0.41
opercular length	**0.73**	**-0.71**
opercular width	**1.13**	0.09
rib strength	0.19	**0.85**
no. ribs	0.27	-0.50
constriction	0.04	-0.13
aperture sculpture	-0.05	-0.29
parietal scar	-0.31	0.36
spire angle	0.23	0.48
cumulative % of total variance	46.5	65.6

The values in bold indicate the variables that contribute most to the separation of the groups.

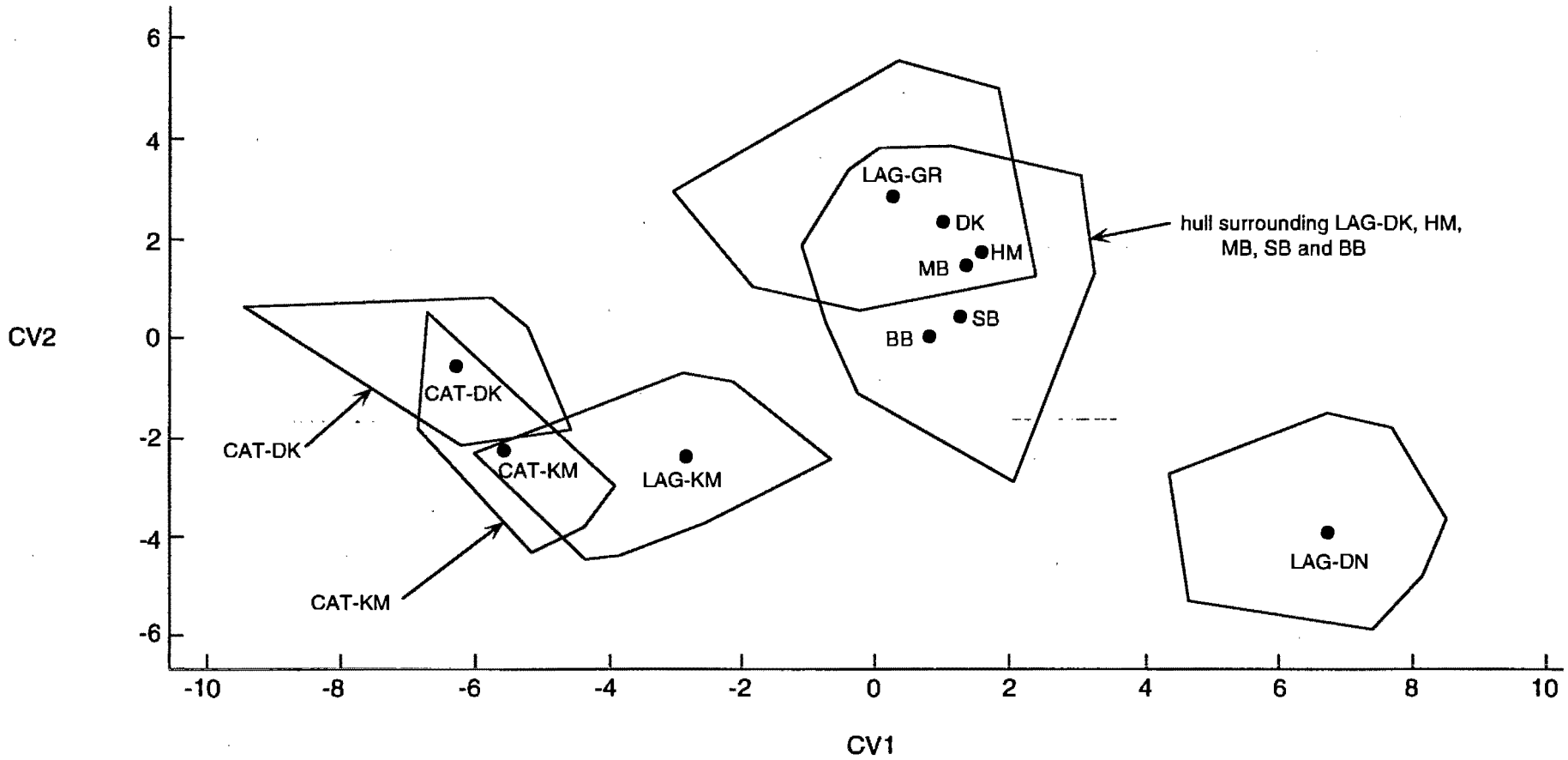


Figure 23. Plot of the first two canonical variables for the populations of *B. lagenaria* and *B. catarrhacta*. To reduce the clutter, five of the *B. lagenaria* populations have been included in a single convex hull, although the centroids for each population are shown.

due to a smaller operculum (both length and width) and slightly longer spire (Fig. 10e-b). The second canonical variable did not separate these two species (Fig. 23).

***B. papyracea, B.* sp. A and *B. cincta limbosa* from the West Coast only**

In her revision of the genus, Orr (1956) synonymized *B. limbosa* with *B. papyracea*, finding it to be an "ecologic form occupying more exposed situations". She also includes with *B. papyracea* the species identified here as *B.* sp. A (the specimen figured in her Plate 19, Fig. 4). Since both *B. c. limbosa* and *B.* sp. A have only been found on the West Coast, it is once again in this region that confusion between species has previously occurred.

The plot of the canonical variables for all species together (see Fig. 6a) showed some overlap between *B. papyracea* and *B.* sp. A, and slight overlap between *B. papyracea* and *B. c. limbosa*. A discriminant analysis of the seven West Coast populations (two *B. papyracea*, three *B. c. limbosa* and two *B.* sp. A) indicated that about 90% of the animals were correctly identified (Table 25a), but as with the results discussed above, most of the incorrect identifications were intraspecific. Four snails were identified as being more similar to a population of another species. As was found when *B. c. limbosa* and *B.* sp. A were compared (see Table 22a), no individuals from either of these two species was found to be more similar to the other. This was not the case with *B. papyracea*. Two PAP-BB individuals were allocated to another species, one each to *B. c. limbosa* (the BB population) and *B.* sp. A (BO). One LIM-BB animal was placed with PAP-BB, and one A-BO animal was identified as being more similar to PAP-BO (Table 25a). Three of the between-species misidentifications occurred between populations from the same locality. Thirteen variables were found to be useful in distinguishing the populations (Table 25b).

A plot of the first two canonical variates (accounting for 79% of the total variance) is shown in Fig. 24. The separation of the *B. c. limbosa* and *B.* sp. A populations was essentially the same as was seen in Fig. 21, with slight overlap of the LIM-BB and A-BO populations. The two *B.*

Table 25. Discriminant and canonical variate analyses of *B. papyracea, B. cincta limbosa* a *B*. sp. A. (a) Jackknifed classification, showing the percentage of correctly classified snails for each population, and the number of cases classified into each species. (b) the standardiz coefficients for the canonical variables.

(a) Jackknifed classification

		no. of cases classified into species			
population	%correct	PAP	LIM	A	n
PAP-BB	90.0	28	1	**1**	30
PAP-BO	88.4	43	0	0	43
LIM-BB	94.1	**1**	16	0	17
LIM-OK	96.3	0	27	0	27
LIM-KM	96.9	0	32	0	32
A-GR	82.6	0	0	23	23
A-BO	83.3	**1**	0	35	36
total	89.9	73	76	59	208

The values in bold indicate the numbers of animals placed into the wrong species.

(b) Standardized coefficients

	canonical variables	
	1	2
variable		
shell width	0.99	0.51
spire height	-0.26	**2.20**
aperture length	0.27	0.87
shell thickness 1	-0.59	-0.29
shell thickness 2	-0.19	0.21
body weight	**-2.30**	-0.81
shell weight	**2.01**	**-2.19**
opercular length	0.80	0.00
opercular width	-0.91	-0.31
no. ribs	0.20	0.14
constriction	0.54	-0.14
parietal scar	-0.05	-0.35
spire angle	-0.53	0.05
cumulative % of total variance	58.5	79.1

The values in bold indicate the variables that contribute most to the separation of the groups.

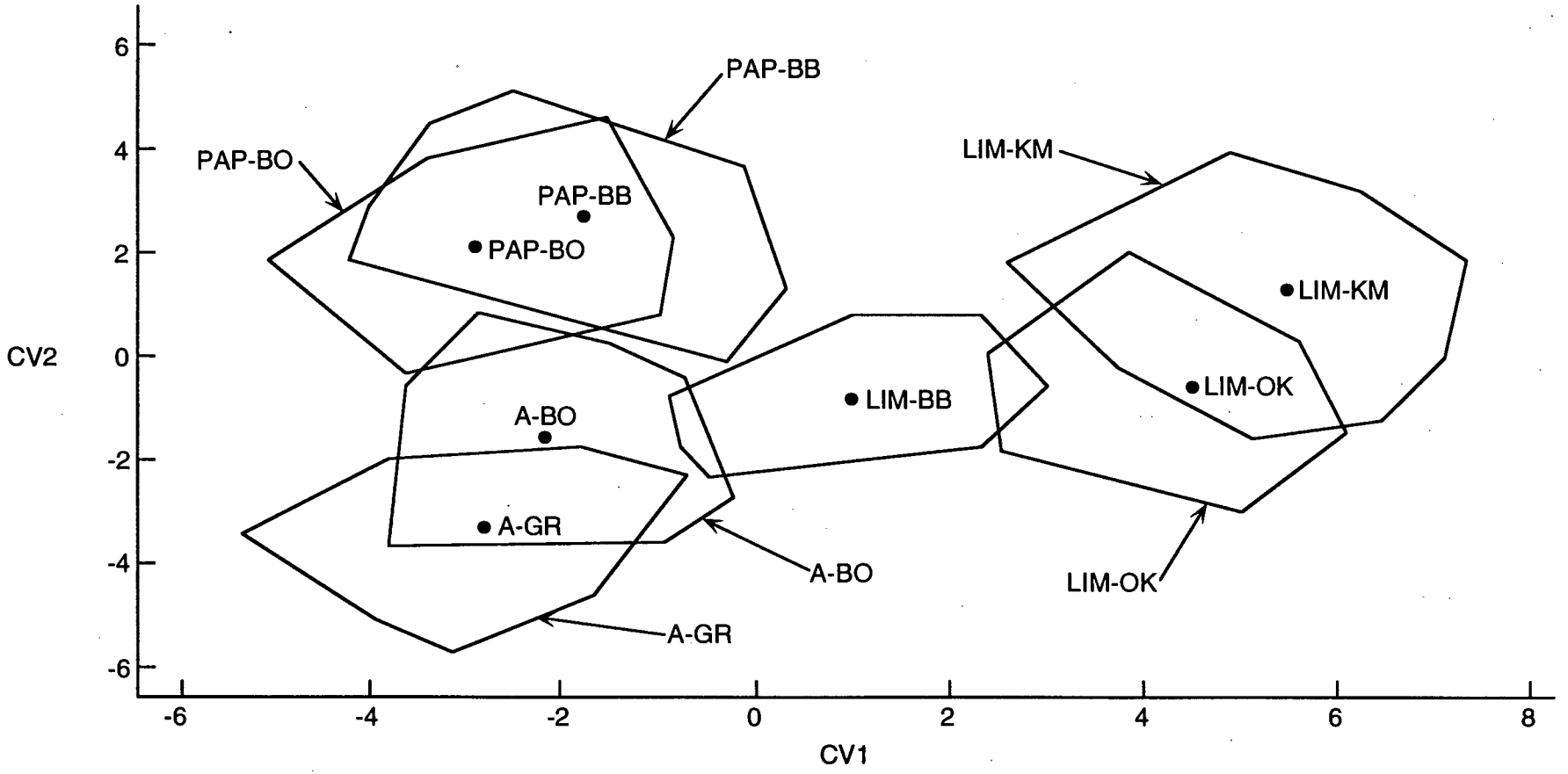


Figure 24. Plot of the first two canonical variables for the West Coast populations of *B. papyracea*, *B. cincta limbosa* and *B.* sp. A.

papyracea populations were not separated from *B.* sp. A along the first axis, which was mainly due to the contrast between body and shell weights (Table 25b). This axis separated the *B. c. limbosa* populations, although LIM-BB not completely, from the other two species. The *B. c. limbosa* populations had heavier shells and generally lighter bodies (Fig. 10d). *B. papyracea* and *B.* sp. A were almost completely separated along the second axis, which contrasted shell weight and spire length (Table 25b). *B. papyracea* were higher spired (Fig 10b) with lighter shells (Fig. 10d).

DISCUSSION

WITHIN POPULATION VARIATION

The amount of morphometric variation within a population can be influenced by two sources - differences between individuals of the same sex, and inter-sexual differences. In only eight of the 29 populations sampled were there no significant differences between the sexes for any of the variables, four of these being *B. lagenaria* populations. The females were larger than the males in 20 of the 21 populations exhibiting inter-sexual differences. Sexual dimorphism has been reported in a number of littorinid species (Struhsaker, 1968), as well as in a number of other species (Fretter, 1984), with females usually being larger than the males. The relative variability, as measured by the coefficient of variation (Table 26) indicated that for most of the populations, there were similar levels of variability within the males, within the females, and with sexes combined. This implies that both sexual differences and within-sex variation influence the amount of within-population variation.

To put the sexual differences into perspective relative to geographical differences, the discriminant analyses were run with the males and females as separate groups. The results for all of the species showed that although the sexes could be distinguished with a relatively high percentage (averaging 71%), there was considerable overlap between them. Of the incorrectly identified animals, over 70% of cases were allocated to the correct population. Therefore, although significant differences between the sexes were present, these were small relative to the differences between the populations.

Table 26. Summary of the coefficients of variation for each of the species. The values are the means for the populations, (a) with the sexes combined, (b) for males only and (c) females only. Shell length and width have been used as representatives of the size variables.

		Species						
variable	group[1]	PAP	CIN/LIM	PUB	LAG	A	CAT	B
shell length	m+f	0.141	0.131	0.087	0.092	0.103	0.113	0.086
	m	0.133	0.118	0.074	0.099	0.095	0.103	0.060
	f	0.131	0.111	0.076	0.080	0.080	0.094	0.096
shell width	m+f	0.124	0.122	0.097	0.092	0.104	0.106	0.075
	m	0.113	0.117	0.084	0.098	0.076	0.101	0.068
	f	0.113	0.099	0.076	0.080	0.076	0.087	0.079
shell thickness 1	m+f	0.309	0.379	0.296	0.352	0.308	0.297	0.310
	m	0.283	0.340	0.205	0.356	0.282	0.298	0.252
	f	0.327	0.385	0.309	0.335	0.292	0.332	0.456
shell thickness 2	m+f	0.367	0.374	0.320	0.312	0.303	0.284	0.273
	m	0.349	0.297	0.232	0.296	0.296	0.306	0.265
	f	0.386	0.372	0.303	0.310	0.292	0.278	0.299

[1] m+f = sexes combined; m = males only; f = females only

INTRASPECIFIC MORPHOLOGICAL DIFFERENTIATION

Variation between species must be considered in the context of variation within a species, especially when there is no pelagic larval stage and adult dispersal is also limited. *Burnupena* spp. lay eggs on the substratum, the young snails hatching as miniature adults (Bokenham et *al.*, 1938), and although little is known about the extent of dispersal by adults, Bokenham et *al.* (1938) suggest that they are probably rather sedentary. Studies of other dog whelks indicate that the adults are also sedentary (Hughes, 1972 in Day & Bayne, 1988). In such cases, plasticity of shell morphology can result in considerable variation within a species along environmental and geographic gradients (Phillips et *al.*, 1973; Spight, 1973; Kitching, 1976; Crothers, 1983; Boulding et *al.*, 1993).

The previously recognised species *B. cincta* and *B. limbosa* can usually be distinguished morphometrically, as was shown in the canonical variate analysis of all species (Fig. 6a). The main character that is used to discriminate between these two species in the field is the presence or absence of ribs: *B. cincta* generally has fairly strong ribs, whilst *B. limbosa* has either no ribs or a few weak ribs on the lower half of the body whorl only (Fig. 5a and 11a). In a preliminary investigation (Dempster, 1986; Grant et *al.*, 1988) it was suggested that *B. cincta* and *B. limbosa* may constitute a single species complex with two shell forms: a ribbed shell found in the warmer waters of the South Coast, and a smooth shell found in the cooler waters of the West Coast. As noted, the strength of the ribs played an important role in the separation of the species along the first canonical axis, and also had a fairly large influence on the separation along the second axis (Table 5b). When the qualitative variables were excluded from the analysis there was almost complete overlap between *B. cincta* and *B. limbosa* (Fig. 6b) and a higher percentage of misidentification occurred (Table 5c). A similar pattern was observed when the discriminant and canonical analyses were performed on all of the populations of *B. cincta* and *B. limbosa* (Table 16, Fig. 13a-b). The results of the cluster analysis based on the quantitative variables only (Fig. 8b) also revealed the similarity of these two species. Furthermore, the outcome of the allozyme electrophoresis (see Chapter 3) indicated that these two species were genetically very similar. It can be seen from Table 1 that the three *B. limbosa* populations were collected on the West Coast (BB,

OK and KM), whilst the five *B. cincta* populations were collected from the Western Overlap (CR, AF and DK) and South Coast (MB and PE). Thus these two taxa appear to occupy different geographic regions. It was for these and other reasons argued below (page 68), that I considered *B. limbosa* to be a subspecies of *B. cincta*.

Within each of the species (except for *B.* sp. B where only one population was sampled) there was variation in the mean shell length between populations (Fig. 9). However, with two notable exceptions, the population means fell within a range characteristic of each species. The two exceptions were the *B. papyracea* Castle Rock and *B. lagenaria* Durban populations which had much smaller snails. The reasons for the small size of the PAP-CR snails are unknown as was discussed in the results for this species. For *B. lagenaria*, it is possible that small size of the LAG-DN individuals is because this site is towards the end of the species' range and the distance between this site and the next site sampled was great (approximately 900 km).

In all of the species, differences in shell size were significant ($p < 0.05$) in almost 80% of the comparisons made between the populations. There were fewer significant differences in the comparisons between populations for shell thickness, although over 35% of them were significant in any one species. Relatively large amounts of inter-populational variation were also seen in the ratios indicating shell shape (Fig. 10a,b,e), relative shell and body weights (Fig.10d) and relative shell thickness (Fig. 10c). The frequency histograms of the qualitative variables (Fig. 5) also showed that, apart from the two rib variables (Fig. 5a-b), there were high levels of intraspecific variation in all of the species, as indicated by the number of states represented for each character.

The within-species discriminant and canonical variate analyses can be used to indicate the amounts and patterns of variation in each species. The results of the discriminant analyses are summarized in Table 27. The percentage of individuals allocated to the wrong population ranged from 5% for *B. catarrhacta* to 17% for *B.* sp. A. However, the implication is that the higher the percentage of individuals that were allocated to the wrong population, the less reliable the function was in distinguishing between the populations and therefore the more similar the populations. In six

Table 27. Summary of the within-species discriminant analyses. (a) Percentage of individuals incorrectly allocated for each species: (b) regional misallocations for *B. cincta* and *B. lagenaria,* showing the number of individuals misallocated. The arrows indicate the region from which these individuals came from.

(a)

	% individuals allocated to wrong:	
species	population	region
B. papyracea	7.0	1.4
B. pubescens	11.3	0.0
B. cincta	13.1	2.7
B. sp. A	16.9	-
B. lagenaria	11.2	4.6
B. catarrhacta	5.1	5.1

(b)

	West coast	Western overlap	South coast
B. cincta		7 <-- sc	
B. lagenaria	1<-- wo	wc --> 4	
		2 <-- sc	wo --> 2

of the 29 populations (PAP-AF, PUB-RE, PUB-PE, LAG-KM, LAG-DN and CAT-DK) all of the individuals were correctly identified, indicating that at least one attribute of these populations was different enough to be able to distinguish individuals belonging to that population from all other populations of the species. The PAP-AF population was the only population (of the 29 sampled) in which the shell width was less than half the shell length, and the spire was more than half the shell length (Appendix A and Fig. 10a-b). The two *B. pubescens* populations had very small sample sizes which may account for their apparent uniqueness. The LAG-KM population was quite different from other *B. lagenaria* populations and showed no overlap with other populations on a plot of canonical variables. The differences were due to a much narrower and thinner shell (Fig. 10a,c). and a more acute spire angle than in other populations (Fig. 11f). As mentioned in the introduction, problems were encountered with identifying the species at Kommetjie. The shells of *B. lagenaria* from other localities were generally stubby in appearance, with a short, obtuse spire and a relatively wide shell. In this respect, the Kommetjie population was atypical. The LAG-DN population was also morphologically very different from the other *B. lagenaria* populations, differing for most of the characters except the aperture and operculum widths (Fig. 10a,e). Individuals from Durban showed the opposite shape to those from Kommetjie, being extremely squat, with a very short spire. The CAT-DK population had a thicker shell and a higher shoulder than the *B. catarrhacta* population at Kommetjie.

Five of the species were collected from more than one region, and the percentage of individuals allocated to a population in incorrect regions are also shown in Table 27a. These values were much lower, indicating that the differences between populations in different regions were greater than were the differences between populations within the same region. Two of the species, *B. cincta* and *B. lagenaria*, were sampled from more than two regions, and the results (Table 27b) showed that when individuals were allocated to an incorrect region, they were always allocated to a neighbouring region. These results imply that not only are there greater morphological differences between regions than within regions, and that the morphological variation within *B. cincta* and *B. lagenaria* shows broad geographical trends.

The plots of the canonical variables for each of the species (Figs 12 to 17) showed the same patterns of morphological differences between the populations as the discriminant analyses. For *B. cincta* and *B. lagenaria*, there also appeared to be more overlap between the South Coast and Western Overlap populations than between the Western Overlap and West Coast populations, even though the two South Coast populations sampled here were geographically far from the Western Overlap populations. This was also reflected in the number of misallocations between regions as shown in Table 27b, with 11 and five cases respectively. This supports the zoogeographic regions distinguished by Emanuel et *al.* (1992), who combined the Western Overlap and South Coast regions in their Warm Temperate South Coast region.

The variables which contributed most to the separation between the populations in each species in the canonical variate analyses are summarized in Table 28. In all cases, the set of variables that was most useful in discriminating between populations of one species, was different from those for the other species. No single variable was found to be useful in more than three species, and of the 18 variables, three (shell length, aperture width and constriction) were not found to be important in the discrimination between the populations of any of the species. On the other hand, the variables that accounted for most of the within-species variation in the principal component analyses were very similar in each of the species (Table 17). The first component always represented variation due to size. Variation in the second component was always due mainly to shell thickness, and the third component represented variation in spire height or shell thickness or both. After the third component, the proportion of the total variance explained by subsequent components is usually less than 1%, implying that the other variables contribute almost nothing to the differentiation between the individuals.

High levels of intraspecific variation were clear when the results of the principal components analyses were examined. Shell size accounted for over 80% of the total variance in the shell measurements for most species (Table 17), but there was almost no separation between the populations, in any of the species, along the axis representing size, other than the *B. papyracea* Castle Rock and *B. lagenaria* Durban populations, which were partially separated from the other

Table 28. Variables contributing the most to the separation of the populations in the first two canonical variables

species	canonical variables 1	2
B. papyracea	spire height shoulder height shell weight	shell weight spire height
B. pubescens	shell width shell thickness 2	shell width shoulder height
B. cincta	rib strength no. of ribs shell weight	spire height operculum width body weight aperture length
B. sp. A	spire angle shell thickness 1 parietal scar	-
B. lagenaria	body weight operculum width spire height aperture length operculum length	rib strength operculum length no. of ribs operculum width shell thickness 2
B. catarrhacta	shoulder height body weight shell thickness 2	-

populations of their respective species. Although shell thickness accounted for the next largest proportion of the total variance, accounting for 7% to 15%, this feature was also unable to differentiate between most of the populations. Apart from a few cases of partial separation between some populations, the overlap between the within-species populations was large, implying high levels of variation within populations which mask differences, if any, between the populations. In a survey of multivariate analyses, James and McCulloch (1990) commented that principal components analysis should not be used in a multiple-sample situation, as it then confounds within- and between-group sources of variation. They recommend that in studies of geographic variation, means for each locality will give an appropriate data reduction. An analysis using the means by population from all of the species is shown in Fig. 25. The scatter of the conspecific populations reiterates the finding that within-species variation is high, although the characteristic size range of each of the species is evident from the position of the populations along the axis of the first component (representing overall size and accounted for about 84% of the total variation).

Cluster analyses, using the means for each of the populations, were undertaken firstly using all 18 variables, and secondly using only the six qualitative variables. The dendrograms produced are shown in Fig. 26a-b. The within-species variation as well as regional similarities between the populations of both *B. cincta* and *B. lagenaria* were further illustrated in the dendrogram produced using all of the variables (Fig. 26a). Two major clusters (labelled A and B) were formed, and despite the intraspecific variability, the populations of individual species tended to cluster together. Two exceptions were *B. papyracea* and *B.* sp. A. All *B. papyracea* populations belonged to cluster A except for the PAP-CR population which clustered with the *B. pubescens* populations in cluster B. This position was expected since, as noted, this population had a much smaller mean size than the other *B. papyracea* populations (Fig. 9), and in the plot of the canonical variables (Fig. 18a) was found to substantially overlap the *B. pubescens* AF and CR populations, but only slightly overlapped the *B. papyracea* AF population. The two *B.* sp. A populations were in different clusters: the Groen River population clustered with West Coast *B. cincta limbosa* and *B. papyracea* populations in cluster A; the Bakoven population clustered with two of the West Coast *B. lagenaria* populations in cluster B. This was not expected, since in the discriminant analysis, this species had the largest

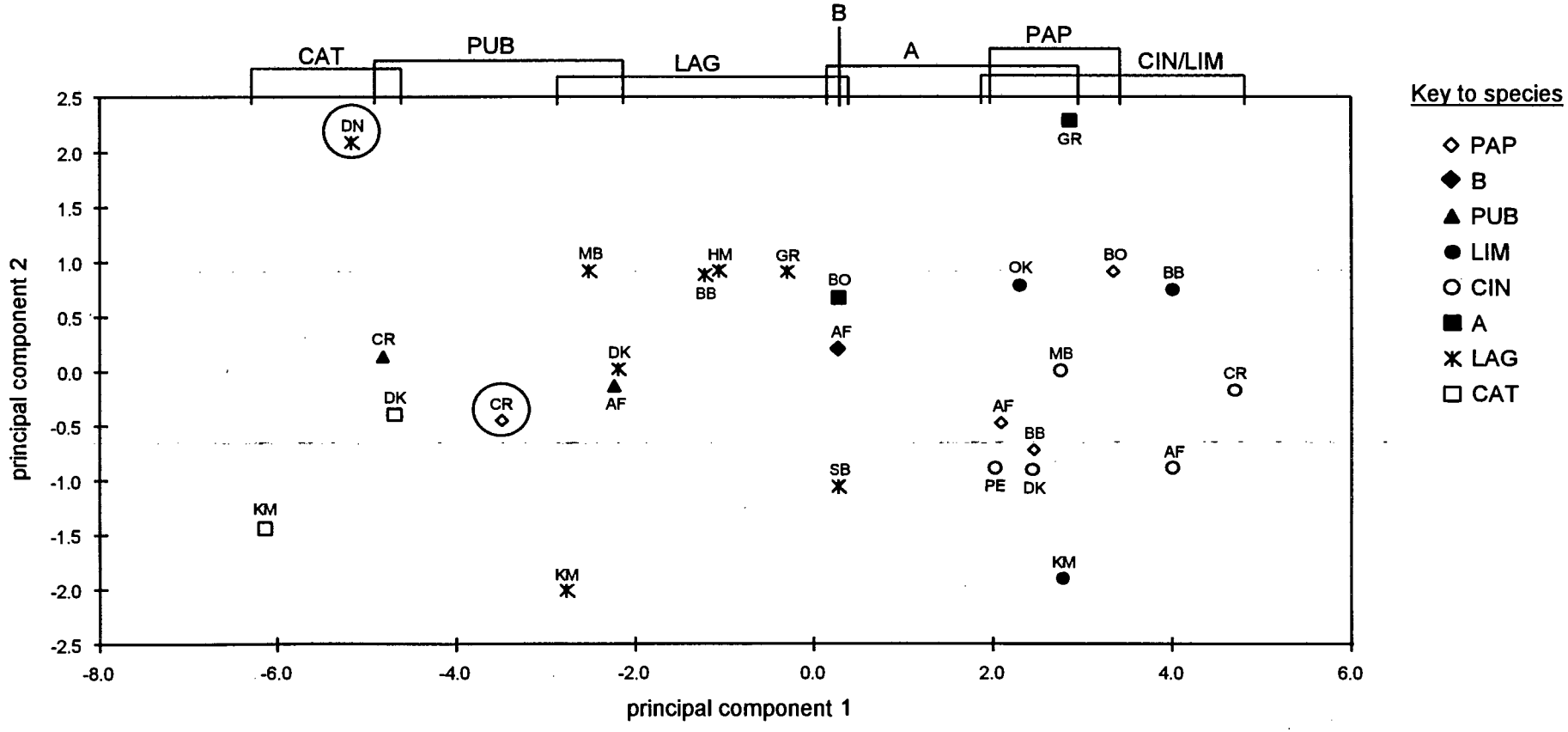


Figure 25. Plot of the first two principal components for the analysis of all populations using means. The positions of the *B. papyracea* CR and *B. lagenaria* DN populations are circled. These two populations are not included in the range of the species shown. See Table 1 for the species and population abbreviations. The first component accounted for 84.2% of the total variation, and represents overall size. The second component accounted for 9.6% and represents shell thickness. The third component accounted for 3.4% of total variance and represents spire height and shell thickness.

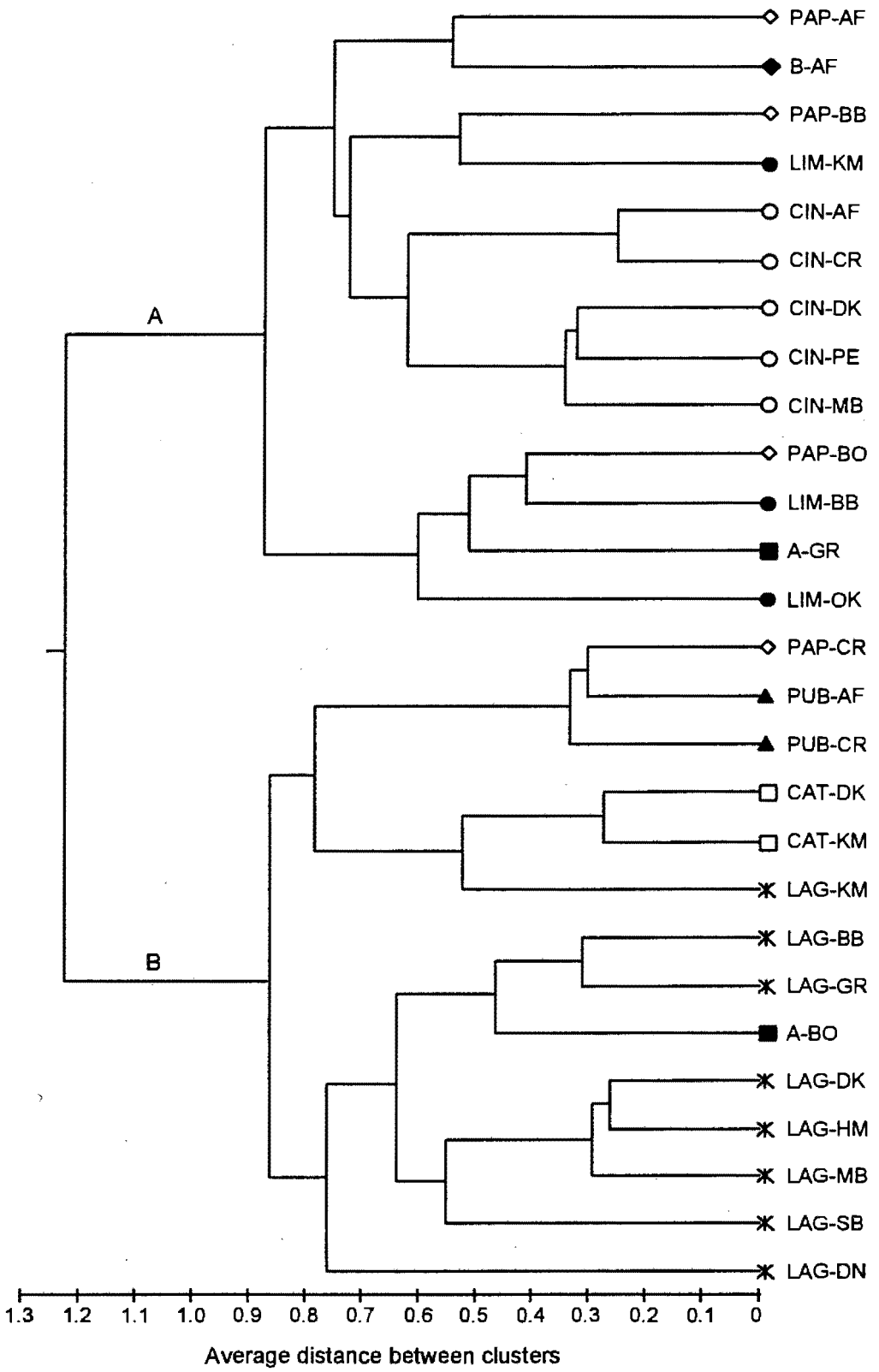


Figure 26a. Dendrogram based on all 18 variables showing the similarities of the populations of *Burnupena* sampled. The symbols for each of the species are as in Fig. 25

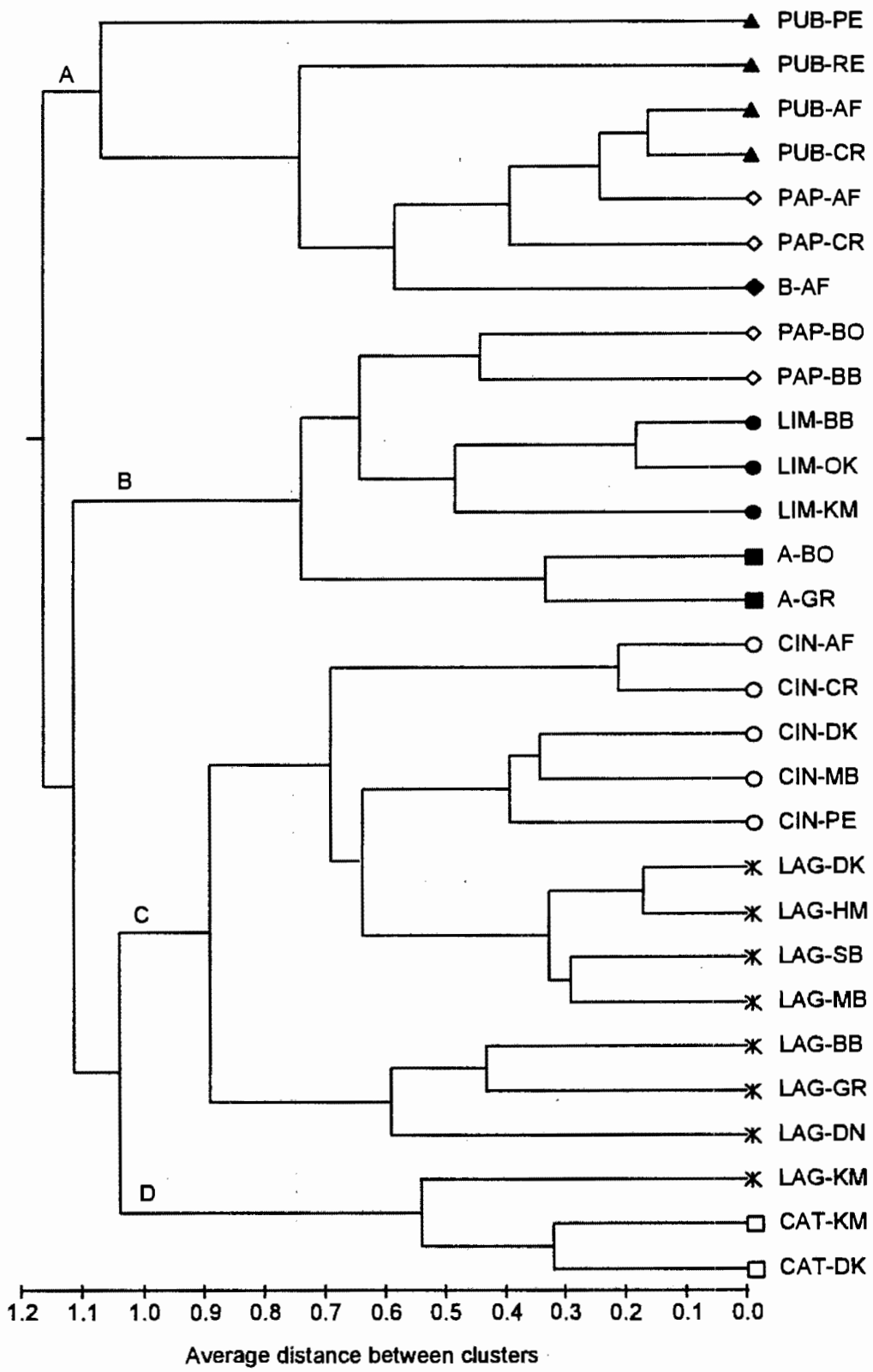


Figure 26b. Dendrogram showing the similarities between all populations sampled based on the six qualitative variables only. The symbols for each species are as in Fig. 25.

percentage of individuals incorrectly allocated between populations (Table 27a) implying great similarity between these two populations. The most likely differences causing this split between these two populations were in shell thickness and weight. Within cluster A, the Western Overlap and South Coast *B. cincta cincta* populations formed one cluster, whilst the three West Coast *B. c. limbosa* populations were in two different clusters. In cluster B, the Western Overlap and South Coast *B. lagenaria* populations were joined together first. These were then joined to a cluster containing two of the West Coast populations, and then the Durban population was added. The Kommetjie population was first linked with the *B. catarrhacta* and a *B. pubescens* /PAP-CR cluster before being united to the other *B. lagenaria* populations. These groupings confirm the greater similarity between populations from the Western Overlap and South Coast regions, relative to the West Coast populations. Apart from *B. catarrhacta* (for which only two populations were sampled), in no species were all of the populations clustered together before linking to other species. A cluster analysis based only on the quantitative variables (Fig. 26b) revealed essentially the same distribution of the populations as was shown in the principal components analysis of all populations, as would be expected.

B. cincta and *B. lagenaria* were well separated in the cluster analysis, yet it is between these two species that many of the difficulties in identification arise. A cluster analysis using only the six qualitative variables revealed why this might be so (Fig. 26b). Although the frequency histograms of these variables showed high levels of intraspecific variation, most of the conspecific populations clustered together. Four main clusters were apparent. In the first (labelled A), the two Western Overlap *B. papyracea* populations, the new species *B.* sp. B, and the four *B. pubescens* populations were joined, although the Port Elizabeth *B. pubescens* population was quite well separated from the other populations. The second cluster (B) included the West Coast populations of *B. papyracea, B.* sp. A and *B. c. limbosa*. The third cluster (C) included all of the *B. c. cincta* populations and all of the *B. lagenaria* populations except the one from Kommetjie. The latter formed the fourth cluster (D) with the two *B. catarrhacta* populations. This implies that the main differences between *B. cincta* and *B. lagenaria* are size and shape differences since these species are well separated in Fig. 26a in the cluster analysis including these quantitative variables but are not so

obviously separated in Fig. 26b on the basis of qualitative variables only. The clustering of the *B. lagenaria* Kommetjie population with *B. catarrhacta* in both dendrograms concurs with comments made above about the 'non-typical' shape of LAG-KM specimens. This similarity was also observed when these two species were analysed together in the species-pairs section, and demonstrates why problems in identification arose at this locality. It can similarly be seen in Fig. 25, which shows the principal components analysis by population means, that the three Kommetjie populations (*B. c. limbosa, B. lagenaria* and *B. catarrhacta*) had the lowest values for the second component (representing mainly shell thickness).

The strength of the shell ribbing was found to be extremely variable within *B. papyracea, B. cincta* and, to a lesser extent, *B. lagenaria* (Fig. 11a). The two West Coast populations of *B. papyracea* were smooth shelled, whilst the two Western Overlap populations had shells that were fairly strongly ribbed. In the discriminant analysis of this species, the two rib variables could not be used since all four populations were invariant. However, they can be used infallibly to assign snails to the correct region. A similar situation was found with the populations of *B. cincta*. As noted above, the West Coast *B. c. limbosa* populations had either no ribs or a few weak ribs on the lower half of the body whorl only, whilst the Western Overlap and South Coast *B. c. cincta* populations generally had strong ribs (although some Mossel Bay and Port Elizabeth snails had weak ribs). Rib strength was the most important variable in terms of contribution in the discriminant and canonical variate analyses of this species.

B. lagenaria showed a similar pattern of shell ribbing. The West Coast populations tended to have no ribs or a few on the lower half of the body whorl, whilst the Western Overlap and South Coast populations mainly had weakly ribbed shells. The exceptions to this trend were Groen River and Durban populations. Most of the shells in the Durban population had ribs on the lower half only, and the Groen River population had a mixture of shell types (Fig. 11a).

The strength of the ribs of *B. pubescens, B.* sp. A and the new species, *B.* sp. B, also fitted this pattern of variation. All of the *B. pubescens* populations had strong ribs and all were collected

in the Western Overlap and South Coast regions. Both *B.* sp. A populations were collected on the West Coast and were smooth. *B.* sp. B from the Western Overlap had weak ribs. However, both of the two *B. catarrhacta* populations had no ribs, although the Dalebrook population was from the Western Overlap region.

In summary, across all species, and with few exceptions, the West Coast populations tend to have smooth shells, whilst the Western Overlap and South Coast populations were ribbed. The only population collected from the East Coast region (LAG-DN) had a few weak ribs on the lower half of the body whorl.

This finding of similar patterns of variation across species indicates that rib strength is likely to be influenced by environmental differences among the different locations. However, it seems beyond doubt that the number of ribs has a genetic basis. In those individuals that had ribs, the number of ribs on the body whorl was consistent in each species (Fig. 11b). Both *B. cincta* (both subspecies) and *B. lagenaria* had few ribs, ranging from about 4 to 9, with the majority having 6 to 8. The West Coast *B. papyracea* populations and all of the *B. pubescens* populations had more ribs on the body whorl, ranging in number from about 10 to 14, with most having between 11 and 13. The new species, *B.* sp. B, which had weakly ribbed shells, usually had about 16 ribs. Obviously, if an individual had a smooth shell, then the number of ribs was scored as none. It seems likely that those West Coast populations of *B. c. limbosa*, *B. papyracea* and *B. lagenaria* which had smooth shelled individuals, were not smooth because they are genetically unable to develop ribs, but rather that their development could be suppressed by environmental conditions. This was one of the reasons that lead to my decision to consider *B. limbosa* a subspecies of *B. cincta*. Phenotypic plasticity in shell sculpture has often been reported in the literature. Palmer (1984) suggested that the expression of spiral sculpture may be controlled by several genes and found strong evidence to suggest that development of sculpture can be suppressed by environmental cues in *Nucella* (=*Thais*) *lamellosa*. In a study of the genetic basis of shell sculpture in *Nucella* (=*Thais*) *emarginata* Palmer (1985) found that a major component of variation in spiral sculpture was inherited in a Mendelian fashion and that sculpture was dominant over lack of sculpture. He also

found that the phenotypic effect of the genes controlling sculpture could be modified by environmental conditions. In a subsequent study, Palmer et *al.* (1990) found that within populations of *N. emarginata*, different forms of spiral sculpture were present, and that in populations from wave-exposed shores spiral sculpture was very weakly developed or absent. In a study on hydrobiid gastropods, Ponder and Clark (1988) found that spiral ribs may or may not be present, and that the number of ribs was also variable. They suggested that the presence of ribbing may or may not be dependent on genetic differences, but may be due to environmental effects, such as salinity and water temperature, on the phenotype. One of the major differences in environmental conditions between the West Coast region and the Western Overlap and South Coast regions is in the sea temperature, with the West Coast being colder. It is possible that this factor may play a role in suppressing the formation of ribs. Struhsaker (1968) showed that extremes in shell sculpture of *Littorina picta*, from smooth to heavily sculptured, corresponded to exposure to wave action, with smooth forms occurring in the most wave-swept areas. It is unlikely that wave action will play an important role for *Burnupena*, since there are no obvious differences in wave action between the West, South and East Coasts. Furthermore, all populations of *B. papyracea*, whether smooth or ribbed, live subtidally, and are in any case usually covered by a bryozoan. It is not possible to say whether the lack of ribs in *B.* sp. A and *B. catarrhacta* are as a result of environmental effects, or whether these species lack the gene/s required to develop ribs.

The results of numerous studies have indicated that much of the intraspecific variation in shells of gastropods appears to be adaptive. Vermeij (1982) showed that *Nucella lapillus* adapted phenotypically by increasing spire height and shell thickness, in response to the arrival of the green crab *Carcinus maenas* on the east coast of North America. Thomas and Himmelman (1988) showed that shell thickness and aperture length in populations of *Buccinum undatum* were influenced by predation. Appleton and Palmer (1988) and Palmer (1990) found that populations of *Nucella lamellosa* and *N. lapillus* respectively produced thicker shells and larger apertural teeth in direct response to the scent of crabs and damaged conspecifics. Studies on species of both *Nucella* (Currey & Hughes, 1982; Crothers, 1983) and *Littorina* (Janson & Ward, 1984; Grahame et *al.*, 1990) have found that exposed-shore snails tend to be squat with large apertures and a large foot, whilst their

counterparts on sheltered shores tend to be elongated, with a thicker shell and smaller aperture. This pattern has been attributed mainly to the differential selective effects of wave action and predation by crabs. Recently, a study by Gibbs (1993) on the progeny of *Nucella lapillus* adults transplanted from an exposed shore to two sheltered inlets, showed that, whilst the transplants had shells typical of exposed conditions (large aperture and light shell construction), the progeny had characters associated with development under sheltered conditions and subject to crab predation (small aperture and robust shell). It has been shown that variation in shell shape in *Nucella lapillus* can be used in the assessment of exposure (Crothers, 1992).

Within each of the species, the populations tend to inhabit similar zones, and the degree of exposure is similar throughout their geographic range. It is therefore unlikely that differences in exposure would explain the variation in shell form between the populations. The populations of *B. cincta* were collected from different habitats, and although there was some apparent separation between the subtidal and intertidal populations in the canonical variate analysis (Fig. 13a), there were no consistent differences in shell form that might be attributed to a particular habitat.

Although phenotypic responses to environmental factors can lead to differences in shell morphology within a species, differences in shell shape between species may also be genetically fixed adaptations to their environment (Grahame *et al.*, 1990). As noted above, all of the species of *Burnupena* tend to inhabit similar zones throughout their geographic range, and Bokenham *et al.* (1938) suggest that their vertical distribution may be as important as their geographic distribution since they are probably rather sedentary. Five of the species occur in the low intertidal or subtidally where the threat of predation is likely to be greatest. Three of these, *B. papyracea*, *B. pubescens* and *B.* sp. B are covered by a bryozoan, which is toxic and affords protection against predation (Barkai & McQuaid, 1988). The other two species, *B. cincta* and *B.* sp. A are not protected in this manner, but have much heavier shells, and *B. cincta cincta* has strong ribs, both factors increasing the force required to crush the shell (Palmer, 1990). The other two species, *B. lagenaria* and *B. catarrhacta* are not generally found subtidally, and have relatively lighter shells. *B. lagenaria* is usually squat and has the second-largest aperture of all the species, and tends to inhabit exposed positions. *B. catarrhacta*, although found as high up the shore as *B. lagenaria*, tends to congregate in crevices and gullies, which may account for their slender shape. However, whilst it is possible that differences in shell shape between *Burnupena* species may be adaptive, this still needs to be investigated.

INTERSPECIFIC MORPHOLOGICAL DIFFERENTIATION

The overall conclusion that can be drawn from the preceding section is that, in all of the species the degree of intraspecific morphological variation is relatively large. This is a common finding in studies of other gastropods (Phillips et *al.*, 1973; Janson & Sundberg, 1983; Chow, 1987; Thomas & Himmelman, 1988; Boulding, 1990; Palmer et *al.*, 1990). The variability in *Burnupena* is manifested both by the quantitative variables (as indicated by the principal components analysis of all of the populations, see Fig. 25), and the qualitative variables (as seen in the frequency histograms, see Fig. 5). The cluster analysis of all of the populations using both types of variables (Fig. 26) also supports this finding. However, differences between some species could be discerned in the principal components and cluster analyses.

Apart from the number of spiral ribs (RIB2), no single variable could be used to unambiguously distinguish any of the species. However, there were a few variables that were more useful in distinguishing between the species than others. These included spire height, shell weight and the strength of the ribs.

The ratios indicating shell shape and relative weights and sizes showed that there were both similarities and contrasts between most of the species. There appears to be two basic shapes: squat, with high shoulder and large aperture, and slender with lower shoulder and shorter aperture. However, there is a continuum of shapes between these two extremes. Most of the *B. lagenaria* and *B.* sp. A shells were similar in shape and fitted the first type; *B. pubescens* and *B. catarrhacta* were also similar and tended to resemble the second type; the other four species generally had shells in between these two extremes.

The frequency histograms of the qualitative variables indicated that apart from the two rib variables, most of the species exhibited a wide range of states, although many were present at low frequencies. Hence there was a great deal of overlap between many of the species for these characters. There was much less within-species variation in the strength and number of ribs, but

only *B.* sp. B could be separated unequivocally from all of the other species by its apparently unique number of ribs (> 14). For all of these variables the species could be grouped into overlapping sets (Table 29). However, for both the ratios and qualitative data sets, although the species could be grouped into those having similar shapes or states for each variable, these groups were not consistent across all of the variables, thereby making it difficult to identify suites of characters which can be used unambiguously to separate the species.

Despite these overlaps between the species, there were significant differences in mean shell length (or at least one size-related variable) between all of the species. As mentioned above, most of the population means were within a range characteristic for each species. However, as with the other variables, these ranges overlapped. Groups of species based on the range of shell length are shown in Table 30, where the range was arbitrarily divided into 5mm intervals. All that can be concluded is that very small adults (<30mm) are not likely to belong to *B. cincta*, whilst the very large animals (>50mm) are not likely to be *B. catarrhacta*, *B. pubescens* or *B. lagenaria*.

In spite of the similarities and overlapping distributions of the species, not all species overlapped all of the other species. Rather, there was a continuum of overlapping ranges with the species at the ends more or less distinguishable. These 'end' species varied, being dependent upon the variable/s considered. This was demonstrated in the results of the discriminant and canonical variate analyses which used the variables that gave the best separation between the species. The fact that about 94% of the individuals were identified correctly indicates that when all of the variables were considered simultaneously, differences between the species were detectable. The means of the canonical variables for each species were relatively well separated from one another (Fig. 6a). Although the qualitative variables group the species differently, they do contribute greatly to the overall separation between the species, as demonstrated by the greater overlap between the species (Fig. 6b) and the lower percentage of correctly identified individuals (82%) when these variables were excluded. This was also evident by the increased distance at which the species were joined in the cluster analysis when these variables were excluded (Fig. 8b).

Table 29. Groupings of species based on group membership for each of the states for the qualitative variables.

Character	State	PAP	B	PUB	CIN	LIM	A	LAG	CAT
rib strength	1: smooth	✓				✓	✓	✓	✓
(RIB1)	2: smooth / weak					✓		✓	✓
	3: weak		✓		✓			✓	
	4: strong	✓		✓	✓				
no. of ribs on body whorl	1: none	✓				✓	✓	✓	✓
(RIB2)	2: few (4 - 9)				✓	✓		✓	✓
	3: intermediate (10 - 14)	✓		✓					
	4: many (> 14)		✓						
constriction	1: none	✓		✓		✓	✓		
(CON)	2:slight	✓	✓	✓	✓	✓	✓	✓	✓
	3: moderate	✓	✓	✓	✓	✓	✓	✓	✓
	4: marked			✓	✓			✓	✓
aperture sculpture	1: smooth			✓	✓		✓	✓	✓
(AS)	2: plicate at edge	✓	✓	✓	✓	✓	✓	✓	✓
	3: plicate internally	✓	✓	✓	✓	✓	✓	✓	✓
parietal scar	1: none	✓	✓	✓	✓	✓			
(PS)	2: pale	✓	✓	✓	✓	✓	✓	✓	✓
	3: dark	✓		✓	✓	✓	✓	✓	✓
spire angle	2: 40° to 50°			✓		✓		✓	✓
(SA)	3: 50° to 60°	✓	✓	✓	✓	✓		✓	✓
	4: 60° to 70°	✓		✓	✓	✓	✓	✓	
	5: 70° to 80°	✓			✓	✓	✓	✓	
	6: > 80°						✓	✓	

Table 30. Percentage of individuals found in each shell length interval for each of the species

species	shell length range (mm)							
	< 26	26-30	31-35	36-40	41-45	46-50	51-55	>55
CAT	12.5	52.5	35.0	-	-	-	-	-
PUB	8.0	36.0	36.0	17.3	2.7	-	-	-
LAG	12.6	18.1	50.7	14.9	3.7	-	-	-
A	-	8.5	18.6	50.9	20.3	1.7	-	-
B	-	-	6.2	62.5	18.8	12.5	-	-
PAP	2.8	7.7	9.9	14.4	23.2	27.6	13.3	1.1
CIN	-	-	3.9	15.0	23.7	21.2	23.2	13.0
LIM	-	-	3.8	19.0	25.3	27.8	16.5	7.6

The results of the discriminant and canonical variate analyses indicated that the eight species could be separated into more or less distinct groups, although without the qualitative variables these groups were less obvious. However, within these groups the species were morphometrically fairly similar, as evidenced by the large amount of overlap between the individuals in the species, and also the allocation of individuals to the wrong species. Although these groupings can be discerned, and differences between the species detected, all of the species in the genus appear to have very similar shell morphologies.

The principal components analysis was unable to differentiate between any of the species (Fig. 7) due to the high levels of intraspecific variation. As discussed above, using the mean values for groups would remove this component and make the comparison between species more meaningful (James & McCullogh, 1990). The results of such an analysis are shown in Fig. 27 and the separation between the species is clear, as is the similarity between *B.* c. *cincta* and *B.* c. *limbosa*, providing further justification for my decision to treat these taxa as conspecific and not retain their original separation into two distinct species. The three axes in Fig. 27 between them account for over 98% of the total variability contained within the quantitative variables used. Each of the three axes separated some of the species from the others. The grouping of the species was completely consistent with that produced by the cluster analysis of the 12 quantitative variables (Fig. 8b).

In terms of the groupings of species based on shell morphology, the various multivariate approaches were inconsistent. The canonical variate analysis using 18 variables (Fig. 6a) produced the following broad groups:

* *B.* sp. B
* *B. papyracea* and *B. pubescens*
* *B. cincta cincta*
* *B. lagenaria, B. cincta limbosa, B. catarrhacta* and *B.* sp. A

The cluster analysis using 18 variables (Fig. 8a) produced two major clusters:

* *B. papyracea, B. cincta cincta, B. cincta limbosa* and *B.* sp. A

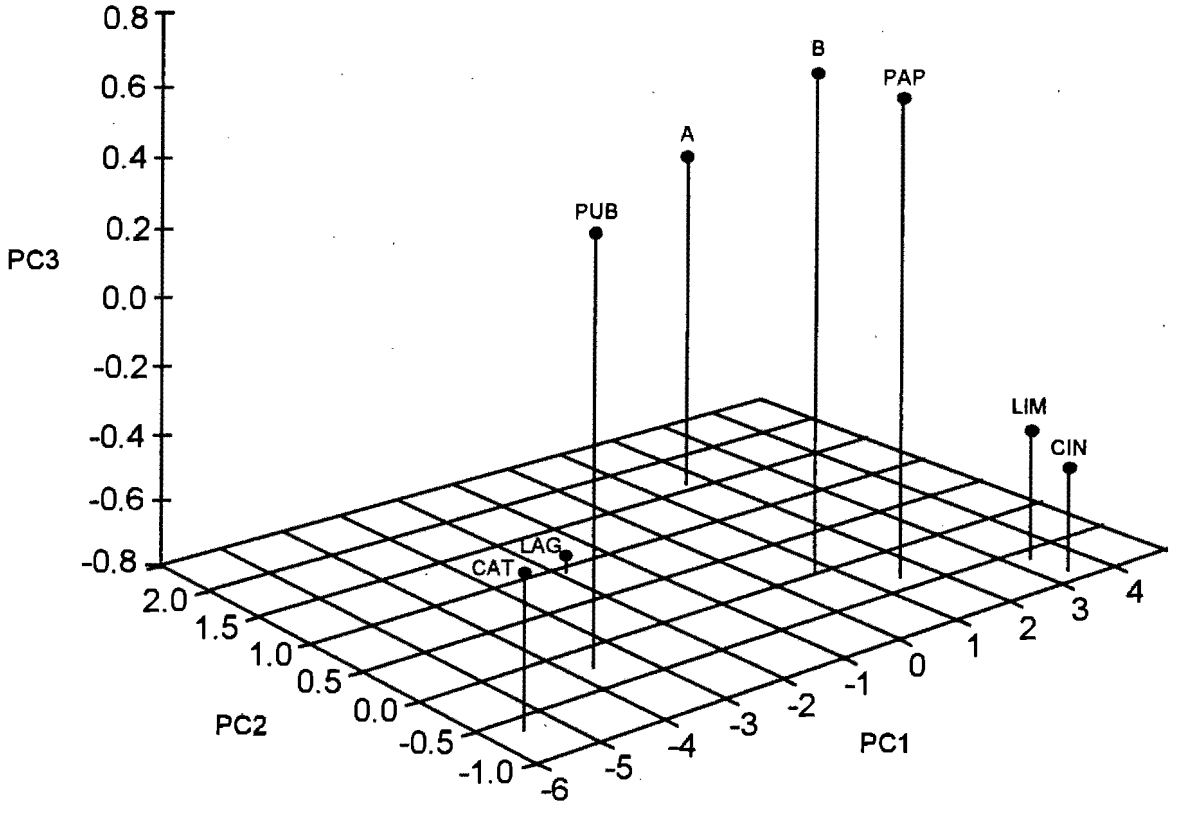


Figure 27. Plot of the first three principal components (PC) using the means for each of the species. The first component accounts for 90.2% of the total variance and represents size. PC2 and PC3 account for 6.0% and 2.5% respectively and both represent shell thickness and spire height.

* *B. pubescens*, *B.* sp. B, *B. lagenaria* and *B. catarrhacta*

When the qualitative variables were excluded the canonical variate analysis (Fig. 6b) produced two main groups:

* *B. cincta cincta*, *B. cincta limbosa*, *B. lagenaria* and *B.* sp. A
* *B. papyracea*, *B.* sp. B, *B. pubescens* and *B. catarrhacta*

whilst the principal components (Fig. 7a-b) and cluster analyses (Fig. 8b) produced the following major groups:

* *B. papyracea*, *B.* sp. B, *B.* sp. A, *B. cincta cincta* and *B. cincta limbosa*
* *B. pubescens*, *B. catarrhacta* and *B. lagenaria*

Although certain combinations do occur more than once, and other combinations do not occur at all, the overall conclusion is that the groups produced using shell morphology are not robust. These results stress the importance of using more than one multivariate approach rather than drawing conclusions about species affinities based on a single method.

No single species, apart from the new species, *B.* sp. B, was easily distinguishable from all of the other species, either historically, or by the canonical analysis based on all species (Fig. 6). However, no species was confused with all of the other species. It has only been between some pairs of species that confusion and overlap have occurred.

Some of the pairs of species previously confused, were clearly separated even when all species were considered simultaneously, and, in all of the groupings produced by the different methods (see above), were virtually never grouped together. These included the following pairs of species:

* *B. cincta cincta* and *B. catarrhacta*
* *B. cincta limbosa* and *B. catarrhacta*
* *B. cincta cincta* and *B. pubescens*

B. c. *cincta* and *B.* c. *limbosa* can both be distinguished from *B. catarrhacta* by shell length, as there was only slight overlap between them in the 31 to 35mm range (Table 30), as well as by relative operculum length and shell weight (Fig. 4f-g). *B.* c. *cincta* can also be distinguished completely from *B. catarrhacta* by rib strength (Fig. 5a), and almost completely by the number of ribs and the intensity of the parietal scar (Fig. 5b,e). *B.* c. *limbosa* can also be separated, for the most part, from *B. catarrhacta* by the aperture sculpture and the parietal scar (Fig. 5d-e). *B.* c. *cincta* and *B. pubescens* can be distinguished by their relative operculum lengths (Fig. 4f) and shell weights (Fig. 4g), and they differ completely in their number of ribs (Fig. 5b), and substantially in the degree of constriction (Fig. 5c).

In the light of this, the earlier difficulties in distinguishing *B. catarrhacta* and *B. cincta* are surprising. However, as noted in the introduction, it would appear that many of the animals identified as *B. catarrhacta* on the West Coast were in fact *B. lagenaria*, and therefore most of the confusion was in reality most likely to have been between *B. lagenaria* and *B. cincta*.

Those species which were not well separated by the discriminant and canonical variate analyses were mostly the same as those species between which difficulties in identification have arisen in the past. In an attempt to discover differences between these problematic species, six species pairs/groups were analysed separately. In all but one of these analyses, some individuals from every species were incorrectly allocated to the other species (summarized in Table 31), and all of the plots of the canonical variables showed overlap between some of the populations from each of the species. Only the comparison between *B. cincta limbosa* and *B.* sp. A showed complete separation between species. They differed in their relative shell widths (Fig. 4a), shell thickness (Fig. 4e) and shell weights (Fig. 4g). They were very similar for all of the qualitative variables except for the parietal scar (Fig. 5e) by which they could be distinguished reasonably well.

Table 31. Summary of the six species-pairs discriminant analyses showing the percentage of individuals allocated to the wrong species.

	species pair/group	percentage incorrect
(1)	*B. papyracea*	1.4
	B. pubescens	4.0
	B. sp. B	13.3
(2)	*B. cincta*	2.1
	B. lagenaria	1.5
(3)	*B. cincta limbosa*	0.0
	B. sp. A	0.0
(4)	*B. lagenaria*	1.3
	B. sp. A	2.6
(5)	*B. lagenaria*	1.0
	B. catarrhacta	2.6
(6)	*B. papyracea*	2.7
	B. cincta limbosa	1.3
	B. sp. A	1.7

Although there was overlap between the species in the other five species-pairs analyses, the amount of overlap was small, and the proportion of individuals that were allocated to the wrong species was generally low (Table 31).

For problem cases arising with the *B. papyracea* - *B. pubescens* - *B.* sp. B group, one feature is particularly useful in discriminating between the species. *B. papyracea* lacks a cancellate spire, but both *B. pubescens* and *B.* sp. B have a cancellate spire, although this feature is not as pronounced in *B.* sp. B as it usually is in *B. pubescens*. In addition, the number of ribs on *B.* sp. B was different to that found on *B. papyracea* and *B. pubescens* (Fig. 5b). Since these two rib variables were invariant in each of the populations, they could not be used in the discriminant analysis of these three species (Table 18). Nonetheless, between these two characters, these three species can usually (but not always), be separated. Other characters that were useful in distinguishing these three species were shell weight (Fig. 4g), shell length (Fig. 9), and operculum length and width (Fig. 4f).

One of the most troublesome pairs of species is *B. cincta* and *B. lagenaria*, and they were found to be the most confusing whilst examining specimens at the South African Museum. All but one of the individuals that were allocated to the wrong species were sampled from the West Coast region. The features that distinguish most of the individuals of these two species are: shell length (Fig. 9), shell width (Fig. 4a), spire height and aperture length (Figs. 4b,d), shell weight (Fig. 4g) and parietal scar (Fig. 5e). Rib strength separates *B. cincta cincta* , but not *B. cincta limbosa* from *B. lagenaria* (Fig. 5a).

As noted, most of the *B. lagenaria* and *B.* sp. A were similar in shape, having a squat shell with high shoulder and large aperture. The summary in Table 31 shows that a small percentage of individuals from both species (West Coast *B. lagenaria* only) were allocated to the other species. Most of the individuals of these two species do however differ in the following features: shell thickness (Fig. 10c), shell weight (Fig. 10d), strength and number of ribs (Fig. 11a-b) and constriction (Fig. 11c).

Difficulties in identification between *B. lagenaria* and *B. catarrhacta* occurred on the West Coast, and as the results of the discriminant analysis of these two species showed, only the *B. lagenaria* Kommetjie population was confused with *B. catarrhacta*. The 'atypical' shape of the LAG-KM snails and the joining of this population with the *B. catarrhacta* populations in the cluster analyses (Fig. 26), have been discussed above in the intraspecific section. However, the LAG-KM population does differ from *B. catarrhacta*, in that it has a slightly shorter spire (Fig. 10b), a larger operculum (Fig. 10e), and differs in the strength and number of ribs (Fig. 11a-b).

The simultaneous analysis of the West Coast populations of *B. papyracea*, *B. cincta limbosa* and *B.* sp. A was performed because Orr (1956) included the latter two species with *B. papyracea*. As with the other species-pair comparisons, there was a small percentage of individuals from all three species that were allocated to the wrong species (Table 31). However, as noted above, no *B. c. limbosa* and *B.* sp. A individuals were confused with one another, and all misallocations involved *B. papyracea*. Although *B. papyracea* and *B. c. limbosa* are similar for most of the variables, they do however, differ in their shell weight (Fig. 10d). *B.* sp. A is not as similar to *B. papyracea* as *B. c. limbosa*, and differs from it in the following features: shell width (Fig. 10a), spire height and aperture length (Fig. 10b) and parietal scar (Fig. 11e)

The results of the discriminant analysis where the discriminant function was used to identify the various type specimens (Table 21), indicated that all of Lamarck's syntypes were found to be morphometrically most similar to their own species, in spite of the fact that the two *B. limbosa* specimens were juveniles (Reeve, 1846; shell lengths = 35.5mm and 34.5mm compared to mean shell lengths for the three *B. cincta limbosa* populations of 42.9mm to 47.7mm). The placement of the type of *Buccinum violaceum* was not so clear cut. Although allocated to *B.* sp. A, the posterior probability for allocating it to *B. catarrhacta* was only marginally lower. Orr (1956) regarded this species as a synonym of *B. cincta*, noting that although it was dark it was "otherwise a typical form of *cincta* often encountered in False Bay...". On the other hand, Barnard (1959) regarded this species as a synonym of *B. catarrhacta*, and although he never saw the specimen, he based his

conclusion on the described colour of the aperture. My own assessment of this type specimen would be to call it a *B. lagenaria*.

These results indicate that although some species can be identified with little difficulty, there will always be those individuals that will be more similar morphometrically to another species. In particular, many of the problems of identification occurred between species on the West Coast. Apart from the comparisons between the *B. papyracea* - *B. pubescens* - *B.* sp. B group and the *B. cincta* - *B. lagenaria* species, where the overlap was only between West Coast populations anyway, all of these comparisons were made between populations from the West Coast. The overall conclusion is that variation within the species is high relative to variation in the genus as a whole, but that distinctions between species do exist. Boulding *et al.* (1993) has similarly noted that the systematics of *Littorina* are difficult, especially when based only on shell morphology, since the degree of morphological variation within species is large relative to the variation between species.

The use of shell characters has thus clarified but failed to resolve fully the systematics of *Burnupena*. In addition to the shell, the molluscan radula is another important hard character which has shown to be a useful aid in distinguishing taxa in many systematic studies (e.g. Cooke, 1917; Peile, 1938, 1939; Kool, 1993 (and references therein)). The following Chapter is concerned with the examination of the radulae of all of the species of *Burnupena*.

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Appendix A. Means of ratios for all populations of *Burnupena* sampled.

population	n*	sw/sl	sp/sl	al/sl	aw/sw	ht/sl	t1/sl	t2/sl	bwt/sl	sw/tsl	ol/sl	ow/sw	ow/ol	aw/al	*
PAP-BB	31	0.54	0.49	0.57	0.50	0.51	0.012	0.026	0.093	0.136	0.37	0.36	0.53	0.48	
PAP-BO	50	0.55	0.49	0.58	0.51	0.52	0.017	0.045	0.092	0.146	0.36	0.36	0.54	0.48	
PAP-CR	42	0.53	0.49	0.56	0.47	0.51	0.012	0.026	0.042	0.060	0.35	0.37	0.56	0.44	
PAP-AF	58	0.49	0.53	0.51	0.49	0.47	0.012	0.029	0.079	0.110	0.34	0.37	0.51	0.47	ht/sl,ol/sl,ow/sw,ow/ol=30
B-AF	16	0.52	0.48	0.57	0.49	0.48	0.014	0.034	0.060	0.093	0.38	0.38	0.52	0.44	
PUB-CR	33	0.53	0.48	0.57	0.46	0.51	0.014	0.032	0.031	0.061	0.33	0.36	0.57	0.43	
PUB-AF	30	0.52	0.49	0.56	0.48	0.49	0.013	0.028	0.045	0.079	0.32	0.33	0.54	0.45	ht/sl,ol/sl,ow/sw,ow/ol=20
PUB-RE	7	0.52	0.47	0.55	0.46	0.48	0.009	0.018	-	-	-	-	-	0.44	t1/sl,t2/sl=4
PUB-PE	5	0.53	0.47	0.58	0.46	0.51	0.009	0.016	-	-	-	-	-	0.42	
LIM-BB	18	0.59	0.45	0.62	0.50	0.52	0.017	0.035	0.097	0.203	0.42	0.38	0.52	0.47	
LIM-OK	29	0.56	0.45	0.62	0.47	0.52	0.013	0.058	0.065	0.237	0.37	0.33	0.51	0.43	ht/sl=10
LIM-KM	32	0.53	0.47	0.60	0.48	0.51	0.008	0.022	0.081	0.218	0.36	0.33	0.49	0.43	
CIN-CR	21	0.53	0.47	0.58	0.48	0.49	0.012	0.033	0.084	0.227	0.39	0.38	0.51	0.43	
CIN-AF	57	0.53	0.48	0.58	0.47	0.48	0.011	0.027	0.095	0.206	0.38	0.37	0.51	0.43	ht/sl,ol/sl,ow/sw,ow/ol=40
CIN-DK	62	0.54	0.47	0.59	0.49	0.49	0.010	0.026	0.085	0.159	0.37	0.39	0.56	0.45	
CIN-MB	45	0.53	0.44	0.62	0.48	0.51	0.014	0.028	0.069	0.196	0.38	0.38	0.54	0.41	swt/sl=40
CIN-PE	22	0.54	0.44	0.62	0.46	0.51	0.012	0.019	0.072	0.161	0.39	0.38	0.53	0.40	
A-GR	23	0.62	0.43	0.65	0.49	0.55	0.025	0.051	0.097	0.197	0.40	0.37	0.57	0.47	
A-BO	36	0.61	0.42	0.66	0.50	0.55	0.017	0.035	0.073	0.122	0.38	0.34	0.55	0.47	
LAG-GR	37	0.64	0.37	0.71	0.52	0.56	0.018	0.032	0.081	0.103	0.39	0.36	0.58	0.47	
LAG-BB	14	0.63	0.37	0.71	0.50	0.57	0.017	0.033	0.058	0.107	0.39	0.35	0.57	0.45	
LAG-KM	29	0.55	0.42	0.63	0.50	0.53	0.010	0.016	0.048	0.083	0.36	0.34	0.52	0.44	
LAG-DK	33	0.59	0.41	0.66	0.49	0.54	0.014	0.027	0.052	0.086	0.38	0.37	0.58	0.44	
LAG-SB	19	0.59	0.42	0.65	0.49	0.52	0.011	0.020	0.069	0.128	0.40	0.38	0.57	0.45	
LAG-HM	17	0.59	0.40	0.67	0.50	0.54	0.017	0.032	0.054	0.099	0.39	0.38	0.58	0.44	
LAG-MB	39	0.57	0.43	0.64	0.48	0.53	0.018	0.032	0.037	0.100	0.36	0.36	0.56	0.43	
LAG-DN	27	0.67	0.34	0.76	0.51	0.62	0.023	0.042	0.028	0.075	0.46	0.39	0.56	0.45	
CAT-KM	18	0.55	0.46	0.61	0.48	0.52	0.010	0.015	0.030	0.056	0.32	0.32	0.54	0.43	
CAT-DK	22	0.52	0.48	0.58	0.49	0.48	0.012	0.024	0.034	0.063	0.30	0.32	0.56	0.44	

Appendix B. Means of qualitative variables for all populations of *Burnupena* sampled.

population	n	rib strength	no. of ribs	constr-iction	aperture sculpture	parietal scar	spire angle
PAP-BB	31	1.00	1.00	1.19	2.87	1.42	3.97
PAP-BO	50	1.00	1.00	1.46	2.98	1.88	4.84
PAP-CR	42	4.00	3.00	1.26	2.32	1.85	3.46
PAP-AF	58	4.00	3.00	1.78	2.83	1.72	3.78
B-AF	16	3.00	4.00	2.06	2.94	1.81	3.00
PUB-CR	33	4.00	3.00	2.06	2.79	1.79	3.27
PUB-AF	30	4.00	3.00	2.03	2.87	2.03	3.40
PUB-RE	7	4.00	3.00	2.71	3.00	1.00	2.57
PUB-PE	5	4.00	3.00	3.60	1.40	1.40	2.80
LIM-BB	18	1.59	1.59	2.28	2.94	2.22	4.56
LIM-OK	29	1.43	1.43	2.62	3.00	2.10	4.76
LIM-KM	32	1.63	1.63	2.63	3.00	1.94	3.53
CIN-CR	21	4.00	2.00	3.19	2.86	1.67	4.62
CIN-AF	57	3.95	2.00	3.14	2.58	1.83	4.37
CIN-DK	62	4.00	2.00	3.13	1.39	1.94	3.68
CIN-MB	45	3.18	2.00	3.27	1.76	2.13	4.00
CIN-PE	22	3.50	2.00	3.55	1.96	1.64	3.59
A-GR	23	1.00	1.00	1.39	2.87	2.57	5.44
A-BO	36	1.00	1.00	1.67	2.97	2.94	4.89
LAG-GR	37	2.18	1.76	2.54	2.32	3.00	5.49
LAG-BB	14	1.86	1.86	3.43	2.64	3.00	4.93
LAG-KM	29	1.93	1.93	2.93	2.66	2.90	2.97
LAG-DK	33	3.00	2.00	3.70	2.03	2.81	4.70
LAG-SB	19	2.38	2.00	3.42	1.84	2.37	4.47
LAG-HM	17	2.65	2.00	3.82	2.00	3.00	4.47
LAG-MB	39	3.00	2.00	3.41	1.64	2.72	4.28
LAG-DN	27	1.96	1.96	3.44	2.74	2.19	5.33
CAT-KM	17	1.00	1.00	2.28	2.33	3.00	2.94
CAT-DK	22	1.05	1.05	3.05	2.05	2.96	2.91

Chapter 2

Evaluation of the radula as a useful taxonomic character in Burnupena

Figure 3a. SEM micrograph of the central and lateral teeth of the radula of *B. papyracea*. Scale bar = 100 μ m.

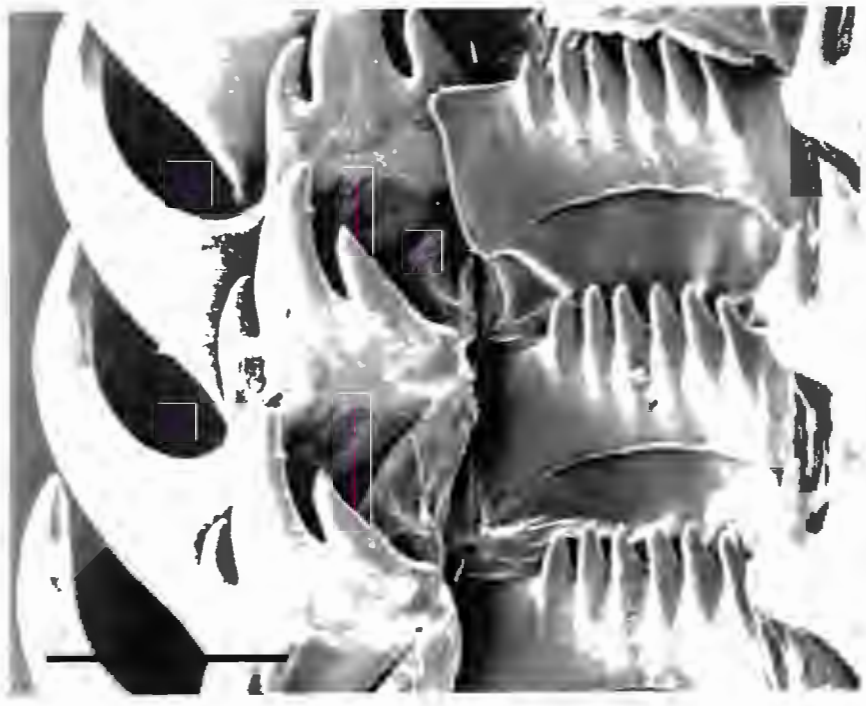

Figure 3b. SEM micrograph of the central and lateral teeth of the radula of *B.* sp. B. Scale bar = 100 μ m.

Figure 3c. SEM micrograph of the central and lateral teeth of the radula of *B. pubescens*. Scale bar = 100 μ m.

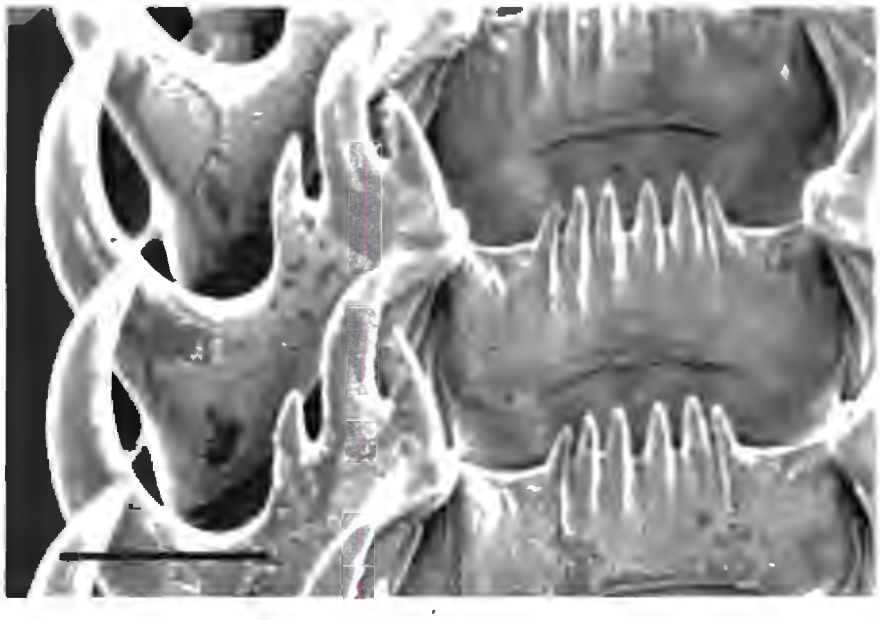

Figure 3d. SEM micrograph of the central and lateral teeth of the radula of *B.* sp. A. Scale bar = 100 μ m.

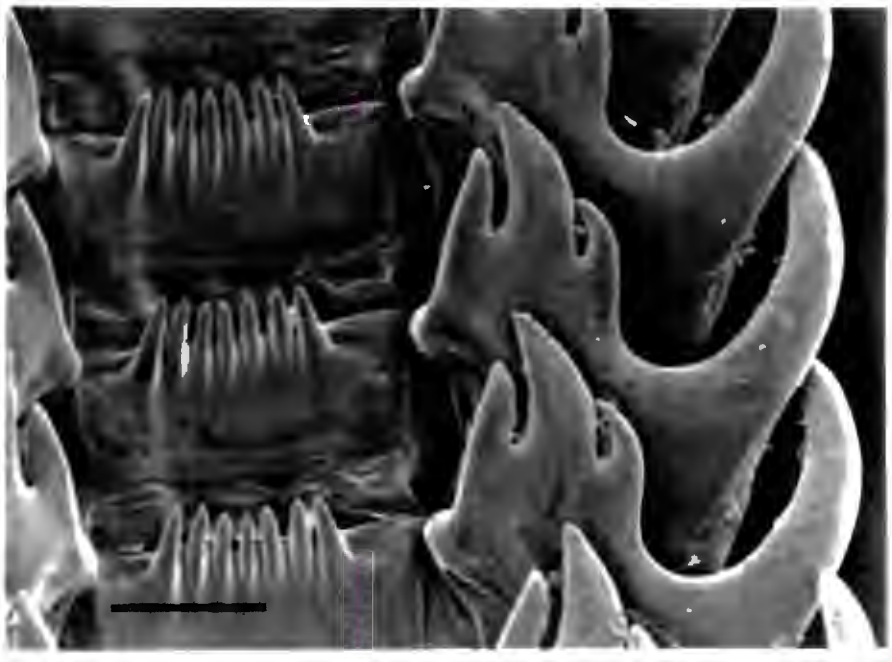

Figure 3e. SEM micrograph of the central and lateral teeth of the radula of *B. cincta cincta*. Scale bar = 100 μ m.

Figure 3f. SEM micrograph of the central and lateral teeth of the radula of *B. lagenaria*. Scale bar = 100 μ m.

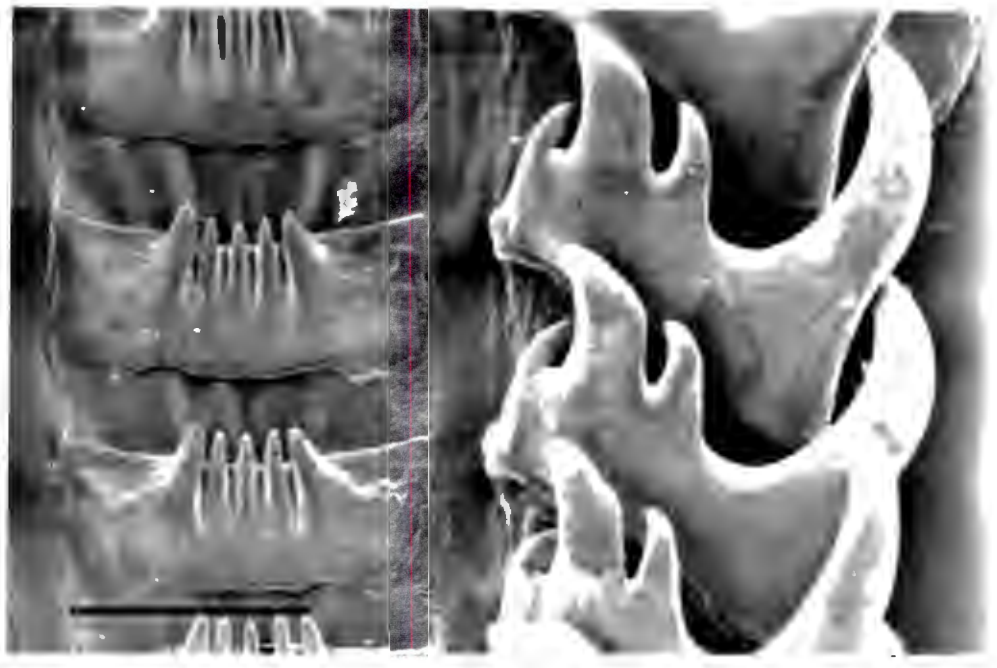

Figure 3g. SEM micrograph of the central and lateral teeth of the radula of *B. catarrhacta*. Scale bar = 100 μ m.

Figure 4a. SEM micrograph of the central plate of *B. catarrhacta*. Scale bar = 50 μ m.

Figure 4b. SEM micrograph of the central plate of *B. c. cincta*. Scale bar = 100 μ m.

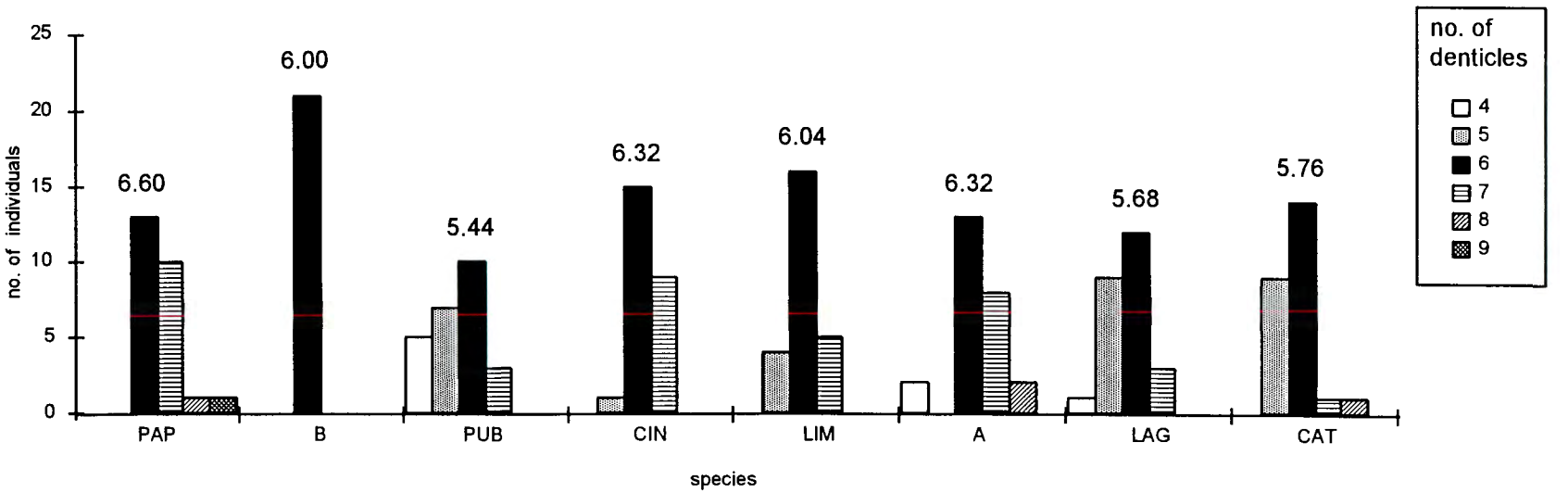

Figure 5. Histogram showing the variation in the number of denticles on the central plate of the radula for each of the species. The mean number of denticles per central plate is given above the bars. N = 25 for each species, except *B.* sp. B for which N = 21. Abbreviations for the species are as follows: PAP = *B. papyracea*; B = *B.* sp. B; PUB = *B. pubescens*; CIN = *B. cincta cincta*; LIM = *B. c. limbosa*; A = *B.* sp. A; LAG = *B. lagenaria*; CAT = *B. catarrhacta*.

noted above, the number of denticles ranged between four and nine, although in all of the species, the most common number of denticles was six. *B. pubescens*, *B. lagenaria* and *B. catarrhacta* tended to have six or fewer, whilst *B. papyracea*, *B. cincta cincta* and *B.* sp. A tended to have six or more denticles. Orr (1956) showed in her figure 2, that the mode for her two species was also six. The numbers of denticles observed in this study are in accordance with those previously reported (Table 1), although Orr (1956) did record as few as three denticles whereas I never noted less than four.

In all species of *Burnupena* the lateral tooth is quadricuspid (Fig. 3), with a very large outer, inwardly curved, cusp, and with the three inner cusps (second to fourth) forming a group, of which the middle one is the largest. The second and third cusps are usually curved, but the fourth tends to be straighter. However, as with the central plate, a number of abnormalities were found (Fig. 6a-b). In 24 of the radulae examined, from all of the species except *B.* sp. B, an additional cusp was present between the outer and the second cusps (Fig. 6a). This cusp was usually present in all of the rows, but not always. In three of the species, namely *B. c. limbosa*, *B. lagenaria* and *B. catarrhacta*, some individuals had the extra cusp on one side only, whilst it was present on both sides in other individuals (Fig. 7). This extra cusp was mostly the same size as the second cusp, although at times smaller, and occasionally no more than a bump.

A conspicuous feature in some specimens was the presence of serrations on the lateral tooth, but in other specimens, although present, they were not so obvious, or consisted only of a few small bumps. Serrations, most commonly on the inner edge of the third cusp and on the outer edge of the fourth cusp (Fig. 8a-b), were seen in all of the species. Fig. 9 shows the proportions of radulae in which the serrations were present (right axis), and the number of serrations on the third cusp (left axis). All specimens of *B. lagenaria*, *B. pubescens* and *B.* sp. B had serrations, whilst in the other species the frequency of individuals with serrations ranged between 35 and 70%, and only in *B. c. cincta* and *B. papyracea* were there more laterals without serrations than with them. The number of serrations on the inner edge of the third cusp ranged between two and six, with the mean number very similar in all of the species, although it was lower for *B. catarrhacta*. However,

Figure 6a. SEM micrograph of a lateral tooth from *B. lagenaria* showing a additional cusp between the outer and second cusps. Scale bar = 100 μ m.

Figure 6b. SEM micrograph of a lateral tooth from *B. papyracea* showing a bifurcation of the third cusp. Scale bar = 100 μ m.

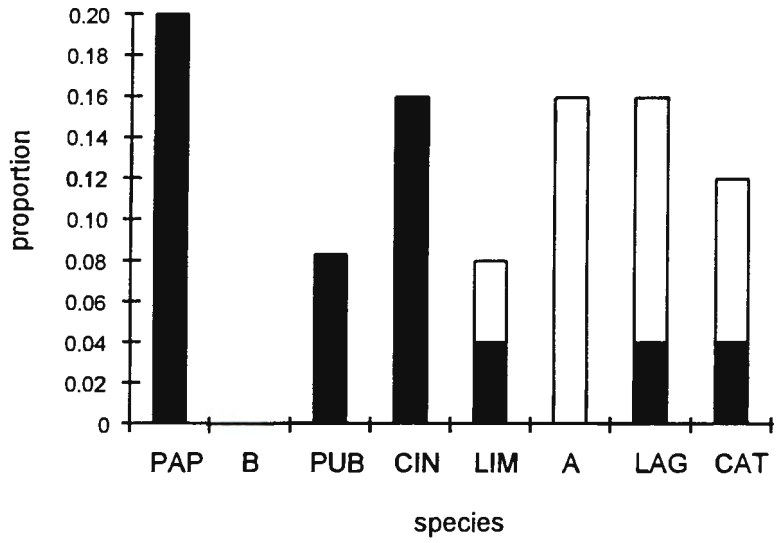

Figure 7. Proportions of radulae with an additional cusp between the first and second cusps. The black bars show the presence of the cusp on one side of the laterals only; the open bars indicate the cusp in the laterals on both sides. Species abbreviations are as in Fig. 5. N=21 for B; N=24 for PUB; N=25 for the remaining species.

Figure 8a. SEM micrograph of a lateral tooth from *B. pubescens* showing strong serrations on the third cusp. Scale bar = 100 μ m.

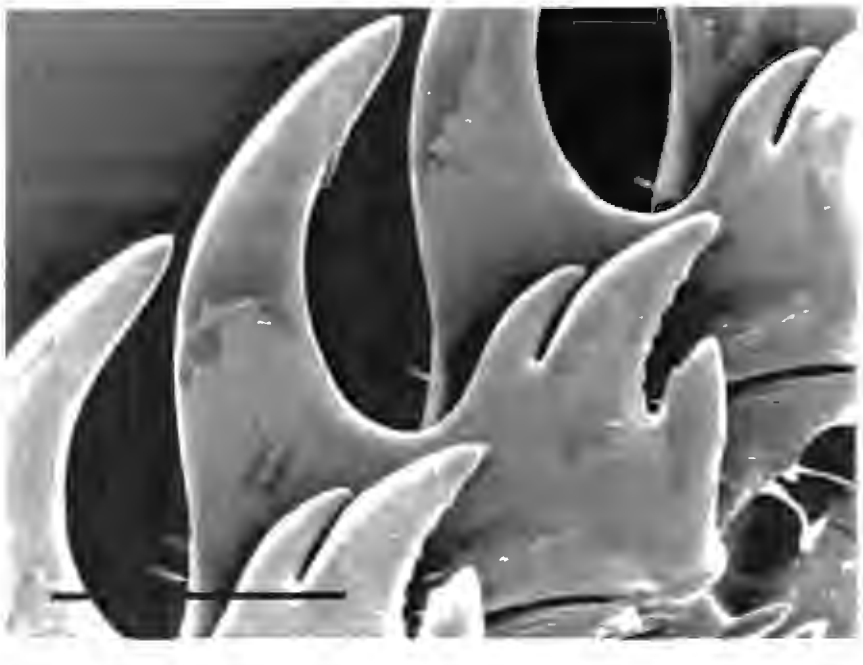

Figure 8b. SEM micrograph of a lateral tooth from *B.* sp. B showing serrations on the third cusp. Scale bar = 100 μ m.

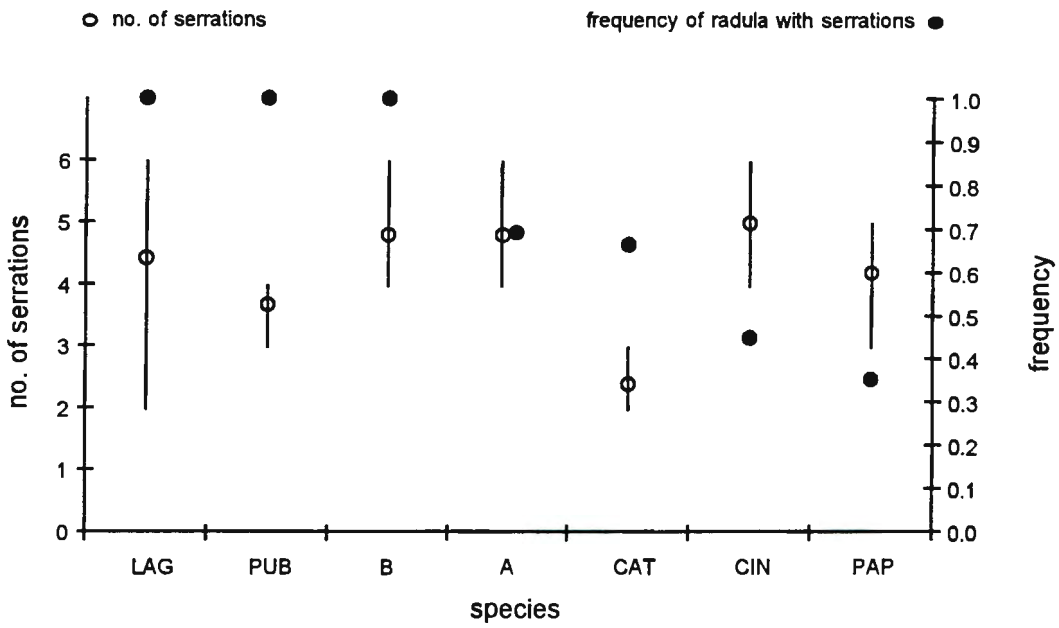

Figure 9. Plot showing the proportion of radula with serrations (right axis; N=25 for all species except B where N=21), and the number of serrations on the third cusp (left axis; N=7 for LAG, N=6 for PUB, N=5 for the remaining species). Abbreviations for the species are as in Fig. 5.

the range for *B. catarrhacta* was completely overlapped by that of *B. lagenaria* which showed the greatest range.

Plots showing the variation in the variables measured from the laterals within each of the species are shown in Fig. 10. For each of the species, the circles to the left show the values obtained from consecutive rows (from both laterals) within one specimen, and indicate the amount of within-individual variation, whilst the data to the right show the mean and range from different individuals. For the latter, the mean per radula was used when consecutive laterals were measured in a single specimen. In many cases, the maximum and minimum values were those found in a single specimen.

The results for CURVE1 (Fig. 10a), indicating the relative curvature of the large outer cusp, show that the within-individual variability is high for most of the species. The means for each species differ slightly between some of the species, but the range indicates almost complete overlap between most of them, so much so, that this character would appear to have no value in distinguishing the species.

Fig. 10b, showing the curvature of the third cusp (CURVE3), again indicates that the variation within individuals is relatively high. There do however, appear to be differences between the species, which can be separated into two groups; *B. pubescens*, *B.* sp. A, *B. lagenaria* and *B. catarrhacta* apparently have cusps which are more curved than those of *B. papyracea*, *B.* sp. B and *B. cincta*. The ranges between these two groups do not overlap, but, given the within-individual variability, it is likely that this would not necessarily always be the case.

The results for the variable WIDTH3, the relative width of the third cusp (Fig. 10c), show a similar pattern to those of CURVE1. The variation within individuals is high for all of the species, and there are essentially no differences between the species. The mean for *B.* sp. A is lower than the minimum values for all of the other species, but its range substantially overlaps those of the other

DISCUSSION

It is clear from the figures of the radulae of *Burnupena* and *Afrocominella*, that there are distinct differences between the two genera. This has long been known (Cooke, 1917; Peile, 1938; Kilburn & Rippey, 1982) and, along with shell characters, lead to the erection of the separate genera (Iredale, 1918). In his section dealing with the family Buccinidae, Barnard (1959) remarked that the retention of the two genera proposed by Iredale depended largely upon the value attributed to differences in the radula. He noted that members of the two genera could always be distinguished by the lateral teeth, but that the central tooth was distinct only in the adults.

Kool (1993, and references therein) noted that intrageneric differences in radular morphology had been "mentioned" in a few studies of gastropods belonging to the subfamily Rapaninae, but that the generic determinations may have been incorrect. In his study on the significance of radular characters as indicators of thaidid phylogeny, Kool (1987) found that there was a high similarity in the radula between three congeneric species of *Nucella*, and also between three congeneric species of *Drupa*. Fujioka (1985) noted that, apart from some minor points, the radular morphology of two species of *Thais* were similar. Within the species of *Burnupena*, the shape of the central plate showed some differences between *B. catarrhacta* and the other species, the former tending to have a straight base, whereas in the other species, the base tended to be slightly concave. However this character was not consistent, even within individuals. Orr (1956) reported that *B. catarrhacta* could be distinguished from the other species of *Burnupena* because of the "rounded central plate". However, Cooke (1917) described the central plate of this species as being slightly arched below, and Peile's (1938) figure of the central plate of *B. catarrhacta* also showed the base to be slightly concave.

The number of denticles on the central plate was variable within all of the species except *B.* sp. B which was invariant, with all individuals examined having six denticles. The number ranged between four and nine, and *B.* sp. B excluded, all of the species had at least three categories.

the second cusp was observed. Further, all individuals examined had serrations on the lateral teeth. There was however, some variability in the number of serrations on the third cusp, and in the three variables measured on the lateral tooth.

Several abnormalities were observed, in both the central and lateral teeth. In his section under the genus *Burnupena*, Barnard (1959) made the comment that malformations and asymmetry of the radula were frequent. I found that the number of denticles on the central plate varied within a single ribbon in one specimen of *B. lagenaria*, and three of *B. pubescens*, with the presence of 'additional' small denticles in some of the rows. Barnard (1959) noted that the central tooth of *B. pubescens* had five to six denticles, with the sixth sometimes minute. This type of abnormality was also reported in a juvenile specimen of *B. catarrhacta* (Peile, 1938).

Abnormalities in the lateral teeth were observed in the form of an additional cusp between the outer and the second cusps. This extra cusp occurred at a frequency of between zero for *B.* sp. B and 0.20 for *B. papyracea*, and in just over 40% of the cases, appeared in the laterals on both sides. Cooke (1917) noted that some of the laterals of *B. cincta* had a similar supplementary cusp, and Peile (1939) noted such abnormalities in four out of five specimens of *B. limbosa*. Although Orr (1956) pooled her data for all of the species except *B. catarrhacta*, she found that 12 out of 68 radulae of her *B. papyracea* complex had the second cusp doubled or tripled on one or both sides of the ribbon, a frequency of 0.14, which is similar to that obtained here. In her remarks on *B. p. papyracea*, Orr (1956) commented that young specimens of *papyracea* that resembled specimens of *B. catarrhacta*, could (together with the colour of the proboscis and the central tooth) be distinguished by the "not infrequent doubling or tripling of the second cusp on the lateral tooth", a character "rarely, if ever, found" in *B. catarrhacta*, although she did not give specific numbers for the latter. In my study however, an additional cusp was found to occur at a frequency of 0.12 in *B. catarrhacta*.

Fujioka (1985) found that seasonal conditions affected the size and shape of the radula in two species of *Thais*, with the rows of teeth produced in winter being malformed. It is unlikely that

species, a finding that supports the conclusion reached by Orr (1956). Although some differences could be detected, these were not consistent, both within and between species. However, the radula is diagnostic for the genus, as there were clear differences in both the central and the lateral teeth from that of its close relative *Afrocominella*.

Kool (1987) found that in 16 species representing nine thaidid genera, radular morphology and anatomy were highly congruent, and that there was no correlation between radular morphology and diet. He suggested that the similarity in the radula of three species of *Nucella* was most likely due to common descent, and that although they had similar diets, it was not necessary to invoke diet as a selective agent influencing radular morphology. He found that five other species that had the same diet as *Nucella* had different radular morphologies. All of the species of *Burnupena* are scavengers, feeding on dead and decaying animal matter, so dietary similarities could explain the similarity of their radulae. However, it is likely that the overall similarity in the radula between the species of *Burnupena* reflects close evolutionary affinities, in view of the large differences between the radulae of *Burnupena* and *Afrocominella*, species of which are also scavengers (Kilburn & Rippey, 1982).

The results of this and the preceding Chapter have revealed that the use of morphological data has clarified but failed to resolve fully the systematics of *Burnupena*. This is a common finding in other studies of molluscs, and has led to extensive use of biochemical techniques, which are independent of, but complimentary to, morphological studies. There have been many studies in which both biochemical and morphological investigations have been used to solve systematic problems in molluscs (e.g. Gould et *al.*, 1975; Dillon & Davis, 1980; Ward & Janson, 1985; Palmer et *al.*, 1990; Boulding et *al.*, 1993). It is in this light that the following Chapter is concerned with a complimentary biochemical approach to the systematics of the genus *Burnupena*.

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Chapter 3

Population structure and species boundaries in Burnupena as elucidated by means of protein electrophoresis

INTRODUCTION

To date, all of the taxonomic investigations of the genus *Burnupena* have been based on a morphological approach, using shell and radular characteristics (Orr, 1956, Barnard, 1959, Kilburn & Rippey, 1982, plus numerous older works). Clearly, the use of morphological data alone has not resolved the problems surrounding the taxonomic status of species within this group.

The limitations of using morphology to resolve many taxonomic problems has led to an extensive use of enzyme electrophoresis as a taxonomic tool in molluscan studies (e.g. Gould *et al.*, 1975; Dillon & Davis, 1980; Ward & Warwick, 1980; Mastro *et al.*, 1982; Davis, 1983; Grant *et al.*, 1984; Hoagland, 1984; Ward & Janson, 1985; Ponder & Clark, 1988; Woodruff *et al.*, 1988; Staub *et al.*, 1990; Knight & Ward, 1991; McDonald *et al.*, 1991; Kemperman & Degenaars, 1992; Boulding *et al.*, 1993). A number of reviews of the systematic uses of electrophoretic data have also been published (e.g. Avise, 1975; Ferguson, 1980; Thorpe, 1982; Buth, 1984; Hillis & Moritz, 1990; Thorpe & Solé-Cava; 1994). Using enzyme electrophoresis, genetic differences between two taxa can be measured over a number of proteins, and reduced to a single measure of genetic similarity or distance. There are several such measures, the most commonly used being those of Nei (1972) and Rogers' (1972). Electrophoretic studies have shown a good relationship between genetic distance (or similarity) and population, specific or generic differences (Avise, 1976; Thorpe, 1982). Conspecifics tend to have lower genetic distances than do congeneric species, which in turn have lower genetic distances than do members of different genera. The relatively large biochemical differences between species make the use of enzyme electrophoresis a very useful tool for identifying members of different species (Avise, 1975).

The basic principle of enzyme electrophoresis is the movement of charged proteins through a supporting medium, such as starch, under the influence of an electric current. Five of the twenty amino acids that make up proteins can be charged, either negatively (arginine, histidine and lysine), or positively (aspartic acid and glutamic acid), depending on the pH. It is the proportion of these

charged amino acids, and the pH of the buffer, that give a protein its overall charge. Proteins with different charges will migrate at different rates, or in different directions, if they have opposite net charges. The rate of movement of the protein will not only depend on its charge, but can also be affected by its size and shape. Larger, bulkier proteins will tend to move more slowly through the medium than smaller, more compact ones.

After electrophoresis, the proteins are visualised, most often by means of specific histochemical staining, where the substrate of the enzyme concerned is brought into contact with the electrophoretic medium. A catalytic reaction leads to the development of a coloured dye, often indirectly through a series of reactions set in motion by the initial reaction between the enzyme and its substrate. The presence of the dye reveals the position of the enzyme.

Differences in mobility between homologous proteins are presumed to represent different alleles, and hence reflect differences in the DNA sequence for that enzyme. However, not all changes in the DNA sequence are detected, since certain base changes do not result in a change in the amino acid, due to the redundancy of the genetic code. Also, since the net charge of the protein is determined by only a few amino acids, many amino acid changes may occur without altering the overall charge. It has been estimated that only about 30% of the possible base changes in the DNA code for amino acids with different charges (Avise, 1975). Apart from the differences in protein mobility due to differences in the protein itself, differences in the physical conditions of the electrophoretic set-up can affect the migration of the protein. These include the pH and ionic concentration of the buffer, concentration of the gel medium, and the amount of heat generated. A great deal of variation has been detected by varying conditions, and by heat denaturization of enzymes prior to electrophoresis (Thorpe, 1982; Murphy *et al.*, 1990). Unless different combinations of conditions are used, it is unlikely that all electrophoretic variants will be detected, and it has been estimated that only about 80% of the alleles can be detected (Woodruff *et al.*, 1988). For most systematic purposes however, the extra work and cost involved would not be justified, except perhaps in the case of recently diverged taxa (Thorpe, 1982). Nevertheless, because of the effects varying conditions have on the detection of genetic variations, it is important to give

details of the electrophoretic conditions for each of the loci examined, to allow meaningful comparisons to be made with other studies. It is obvious, therefore, that since a potentially large amount of variation goes undetected using enzyme electrophoresis, the differences that are detected between taxa are underestimates (Dillon & Davis, 1980). This method does, however, sample a larger proportion of the genome than many of the other biochemical methods, such as nucleotide sequencing and restriction enzyme techniques, provided a large number of loci are used (Nei, 1987).

As stated above, differences in mobility between homologous proteins reflect differences in the DNA sequence. Correct interpretation of the observed banding patterns is therefore necessary to ensure their genetic basis. For most of the enzymes used in electrophoretic studies, the genetic basis has been established, and breeding studies are not necessary (Murphy *et al.*, 1990). There are, however, a number of ways in which the genetic basis can be checked (Grant *et al.*, 1984), and which also ensure that no errors in interpretation have been made. The subunit composition of an enzyme is usually conserved across most taxa, and thus the observed banding pattern of the heterozygotes should conform to the expected pattern. The observed genotype frequencies should fit those predicted by the Hardy-Weinberg equilibrium. Nonconformity could indicate either that the banding patterns have no genetic basis, or that not all of the assumptions of the Hardy-Weinberg equilibrium have been met. Where the enzyme occurs in more than one tissue, the banding patterns produced by the different tissues should be similar. There should be no unexpected phenotypes which may indicate the presence of artefacts or post-translational modifications to the enzyme.

There has been much controversy over the selective neutrality, or lack thereof, of molecular characters (Avise, 1975; Nei, 1987; Moritz & Hillis, 1990). This controversy revolves around the question whether most molecular polymorphisms and molecular changes are due to selection, or to genetic drift of selectively neutral mutations. Are the large amounts of polymorphism found in natural populations maintained by selection (balanced polymorphism) or due to genetic drift of selectively neutral mutations (transient polymorphism)? Although there are examples of balanced

polymorphism, the question is whether or not this is representative of the way in which most polymorphisms are maintained (Ferguson, 1980). However, testing whether molecular evolution is based on selection has proved very difficult. Even in cases where selection appears to be operating on a locus, it is virtually impossible to separate selection at the locus from selection on a group of linked genes which have an advantageous locus. From statistical studies of gene frequency data, Nei (1987) concludes that most polymorphic alleles at protein loci are neutral or almost so, but that there are some polymorphisms that are definitely maintained by selection.

In terms of using enzyme polymorphisms in taxonomy, it is not important whether these polymorphisms are a result of neutral changes or not (Avise, 1975; Ferguson, 1980), and if the changes are neutral, then the probability of convergence will be reduced. However, Murphy *et al.* (1990) note that the use of allele frequencies to study population structure assumes that alternate alleles at a locus are selectively neutral, and Johannesson and Johannesson (1989) note that gene flow, founder effects and non-random mating can be inferred from allele frequency heterogeneity of neutral polymorphisms, whilst selected loci are not as useful in such cases. It has been suggested that, in the absence of evidence for selection, neutrality be used as the working assumption (Allendorf & Phelps, 1981). Further, it is widely assumed that selection will have relatively little overall effect when a number of loci are examined, since most departures from neutrality are thought to be locus-specific (Moritz & Hillis, 1990).

The use of biochemical techniques in conjunction with the more traditional methods of morphology can greatly aid many taxonomic investigations (Avise, 1975; Thorpe, 1982). Indeed, there have been many studies where both biochemical and morphological investigations have been used to solve systematic problems, both in molluscs (e.g. Gould *et al.*, 1975; Dillon & Davis, 1980; Mastro *et al.*, 1982; Ward & Janson, 1985; Hoagland & Davis, 1987; Palmer *et al.*, 1990; Boulding *et al.*, 1993), and in a wide variety of other organisms.

Little is known of the biology of *Burnupena* species. They are scavengers, and are quickly attracted to dead or dying animals. Of importance to this study is the fact that they do not have a

pelagic larval stage. Eggs are deposited in tough, leaf-like capsules, which are cemented to the substratum in domed clusters. The young snails hatch as miniature adults, there being no free-swimming stage (Bokenham *et al.*, 1938). There is evidence to indicate that marine molluscs with limited dispersal, and hence gene flow, show greater population differentiation (Ward & Warwick, 1980; Janson & Ward, 1984; Grant & Utter, 1987; Day, 1990).

A preliminary electrophoretic investigation of five of the six species in the genus (Dempster, 1986; Grant *et al.*, 1988), indicated that *B. papyracea* and *B. catarrhacta* are valid species, but that the three other species examined, namely *B. cincta*, *B. lagenaria* and *B. limbosa* are part of a single species complex. However, the species identifications of most of the populations included in this complex were not certain.

Key Questions

The data obtained from enzyme electrophoresis were used to answer a number of questions regarding the levels of variability and amount of differentiation within and between populations and species of *Burnupena*. Such knowledge can aid decisions regarding the number of species in this genus. For this, it is important to assess the amount of variation within populations and within species in order to put into perspective the differences between species.

(1) Genetic Variation:

(a) How much genetic variation is there within a population?

(b) How much intraspecific genetic variation is there?

(c) Do the levels of variability differ for the different species of *Burnupena*, or do they have similar amounts of intraspecific variation?

(2) Genetic Differentiation:

(a) How much genetic differentiation exists between conspecific populations?

(b) How much genetic differentiation exists between different species?

(c) Is there more difference between populations of different species which occur in sympatry, than between populations of the same species which are well separated geographically?

(d) Do the current nominate morphologically-based species represent genetically distinct entities?

MATERIALS AND METHODS

Samples of *Burnupena* were collected between June 1988 and March 1991, from 17 sites along the coast of South Africa (see Fig. 1 in Chapter 1), fourteen of which were used for the morphometric analyses. These sites were grouped by region (West Coast, Western Overlap, South Coast and East Coast as described in Chapter 1) for some of the analyses. At most of the localities more than one species was collected, giving a total of 38 populations. The regions, sites, distance between sites and numbers of animals collected for each population are shown in Table 1. The population codes used in the section on morphology will be used here: the species and site abbreviations, separated by a dash.

At each site, as many different species as possible were collected and, wherever possible, a minimum of 15 to 20 individuals were collected. As discussed in Chapter 1, the initial species assignments were based on the descriptions given in Kilburn and Rippey (1982) for most of the species, with the addition of two other species referred to as *B.* sp. A and *B.* sp. B. *B.* sp. A has been incorrectly named *B. papyracea papyracea* by Orr (1956) and is illustrated in her Plate 19, Figure 4, but appears to have no valid name although its specific status has been recognised. *B.* sp. B is a newly discovered and still un-named species. Further, as previously discussed in Chapter 1, *B. limbosa* (four populations collected on the West Coast) is considered to be a subspecies of *B. cincta* and in all of the analyses that follows these two taxa were considered jointly.

Animals were kept alive after collection and, in most cases, placed in holding tanks with circulating sea water on the same day. Tissues for enzyme electrophoresis were prepared within a few days of collection. In a few cases, whole animals were frozen at -80°C, and the tissues prepared just before the samples were analysed. A number of other studies follow a procedure of freezing the whole animal prior to electrophoresis (e.g. Gooch *et al.*, 1972; Woodruff *et al.*, 1988; Staub *et al.*, 1990), but in my study I found that for many of the loci, the resolution obtained from

Table 1. Locality data, abbreviations used and sample sizes for the populations of *Burnupena* collected.

Region	Locality Site (abbrev.)		km[2]	Species collected[1] CIN	LIM	PAP	PUB	LAG	A	CAT	B
West Coast (WC)	Groen River	(GR)						20	23		
	Paternoster	(PT)	280		30						
	Blouberg	(BB)	142		18	33		15			
	Bakoven	(BO)	28			45			45		
	Oudekraal	(OK)	4		30[3]						
	Llundudno	(LL)	3			5					
	Kommetjie	(KM)	27		28			22		20	
Western Overlap (WO)	Castle Rock	(CR)	48	25		42	33				1[5]
	A-Frame	(AF)	4	48[3]		37[3]	28[3]				15[3,5]
	Dalebrook	(DK)	13	71[3]				25		27[3]	
	Sparks Bay	(SB)	56	17[4]				15			
	Rooiels	(RE)	7	7[4]		29	7				3[5]
	Pringle Bay	(PB)	7	8[4]							
	Hermanus	(HM)	50			3		17			
South Coast (SC)	Mossel Bay	(MB)	170	50				40			
	Port Elizabeth	(PE)	380	5			10				
East Coast (EC)	Durban	(DN)	900					10			

[1] Species: CIN = *B. cincta cincta*; LIM = *B. cincta limbosa*; PAP = *B. papyracea*; PUB = *B. pubescens*; Lag = *B. lagenaria*; A = *B.* sp. A; CAT = *B. catarrhacta;* B = *B.* sp. B.

[2] Distance in km between successive sites.

[3] Populations consist of individuals collected on different dates, between 5 and 14 months apart.

[4] Populations pooled - site reference is Sparks Bay.

[5] Populations pooled - site reference is A-Frame.

such samples was not as good as that obtained from samples prepared from freshly-collected live specimens.

Three samples of tissue were prepared for each individual: 1) muscle from the foot; 2) mantle, gill and osphradium; 3) digestive gland and reproductive tissue in the top coils of the visceral mass. The tissues were roughly minced on a cold glass plate using a scalpel, and then placed in a 1.5ml eppendorf tube together with an equal volume of 0.01M Tris buffer (pH 8.0). A few grains of acid-washed sand were also added to the tube to facilitate homogenization. Nicotinamide adenine dinucleotide phosphate (NADP) was added to the homogenization buffer in the later samples, as it was found that this greatly improved the resolution of some of the stains. The samples were then frozen at -80°C until required. This procedure of freezing the tissues unhomogenized was also followed by Munksgaard (1990). It was found that tissues stored in this unhomogenized state could be kept for at least six months without any noticeable loss of activity or resolution when compared to samples used within a few days of collection and preparation. On the day prior to analysis, samples were thawed, homogenized using a roughened glass rod attached to a motor, and centrifuged at 10000rpm, at 4°C, for 15 to 30 minutes. The tissues were kept cold at all times. They were then re-frozen overnight, and then thawed the next day and re-centrifuged just before loading onto the gels.

Electrophoresis was performed on horizontal starch gels, following the methods of May *et al.* (1979). Gels were prepared using 42g of Sigma starch and 330ml of gel buffer. Samples were loaded onto the gel by means of 3mm-wide Whatmans #3 filter-paper wicks. After loading, the gels were placed over two trays containing electrode buffer. Sponge wicks were used to make the connection between the gel and the buffer in the trays, which were then connected to a power supply. Electrophoresis was carried out in a 4°C cold room. Gels were electrophoresed at a constant current of 50mA for between three and seven hours, depending on the buffer system used. A marker dye of red food colouring was loaded at intervals in the gel to track the progress of the run, which was stopped when the dye reached the top of the gel.

Table 2. Enzymes, locus abbreviations, apparent subunit structure, buffer systems and tissues used in this study, and numbers of alleles observed at each locus.

Enzyme (EC number)	Locus	Subunit structure	Buffer[1]	Tissue[2]	No. of alleles
Arginine kinase (2.7.3.3)	*ARK*	monomer	1	F	6
Aspartate aminotransferase	*AAT-1*	dimer	2	Dg	4
(2.6.1.1)	*AAT-2*	"	4	M	3
Diaphorase (1.6.2.2)	*DIA-1*	monomer	1/3	F	2
Glucose-6-phosphate dehydrogenase (1.1.1.49)	*G6PDH*	-	6	Dg	2
Glucose-6-phosphate isomerase (5.3.1.9)	*GPI*	dimer	1	F	8
Glyceraldehyde-3-phosphate dehydrogenase (1.2.1.12)	*GAP*	tetramer?	2	Dg	3
Hexokinase (2.7.1.1)	*HEX-3*	dimer	6	F	6
Isocitrate dehydrogenase	*IDH-1*	dimer	2	Dg	3
(1.1.1.42)	*IDH-2*	"	4	M	3
Lactate dehydrogenase (1.1.1.27)	*LDH*	-	1	F	1
Leucine aminopeptidase (3.4.11.-)	*LAP*	monomer	3	F	5
Malate dehydrogenase	*MDH-1*	dimer	4	M	4
(1.1.1.37)	*MDH-2*	-	4	M	3
	MDH-3	dimer	4	M	3
Malic enzyme (1.1.1.40)	*ME-1*	-	5	M	1
	ME-3	-	5	M	2
Mannose-6-phosphate isomerase (5.3.1.8)	*MPI*	monomer	5	M	6
Octopine dehydrogenase (1.5.1.11)	*ODH*	monomer	1	F	7
Peptidase 1 (Glycyl-leucine) (3.4.-.-)	*GL*	dimer	6	F/Dg	6
Peptidase 2 (Phenylalanine -proline) (3.4.-.-)	*PHP*	dimer	6	F/Dg	5
Phosphoglucomutase (2.7.5.1)	*PGM*	monomer	1	F	5
6-Phosphogluconate dehydrogenase (1.1.1.44)	*PGD*	dimer	2	Dg	9
Sorbitol dehydrogenase (1.1.1.14)	*SDH*	tetramer?	1	F	4
Superoxide dismutase (1.15.1.1)	*SOD*	-	5	M	1

[1] see text for details.
[2] F=foot; Dg=digestive gland; M=mantle, gill and osphradium.

After electrophoresis, the end strips of the gel that were covered by the sponge wicks were cut off, and the gels were sliced horizontally into slices using nylon fishing line and 1mm or 1.2mm thick perspex spacers. The slices were then either placed in a staining container, for liquid stains, or onto a white perspex board for stains using agar overlays. The histochemical staining protocols followed those of Harris and Hopkinson (1976) and Shaw and Prasad (1970). Most of the stains were incubated, in the dark, at 37°C. A total of 32 enzymes encoding at least 40 loci were screened initially. However, not all of these of loci were resolvable, and the banding patterns of a few were not interpretable, due to the presence of two or more overlapping loci.

The following six buffer systems were used to assay the 20 enzymes encoding 25 loci that were used:

(1) Electrode (pH 8.0) LiOH 0.06M, boric acid 0.3M; gel (pH 8.7) tris 0.03M, citric acid 0.005M, LiOH 0.006M, boric acid 0.03M (Ridgway *et al.*, 1970).

(2) Electrode (pH 6.1) citric acid 0.04M, n-(3-aminopropyl) morpholine to desired pH; gel (pH 6.5) 1:19 dilution of electrode buffer, plus n-(3-aminopropyl) morpholine to desired pH (Clayton & Tretiak, 1972).

(3) Electrode (pH 8.1) boric acid 0.3M, NaOH 0.06M; gel (pH 8.7) tris 0.076M, citric acid 0.005M (Poulik, 1957).

(4) Electrode (pH 6.9) tris 0.15M, citric acid 0.05M; gel (pH 6.9) 1:15 dilution of electrode buffer (Whitt, 1970).

(5) Electrode (pH 8.6) tris 0.18M, boric acid 0.1M, NaEDTA 0.004M; gel (pH 8.6) 1:4.4 dilution of electrode buffer (Markert & Faulhaber, 1965).

(6) Electrode and gel (pH 9.0) tris 0.087M, boric acid 0.0087M, NaEDTA 0.001M (Ayala *et al.*, 1972).

The loci assayed, the buffer system and tissue used for each locus, and the enzyme subunit structure are shown in Table 2. Alleles were designated by their mobility relative to the most common allele (designated 100) in the *B. cincta* population at Dalebrook, which was used as a

reference population, and a few individuals of which were included in each gel. Alleles which migrated cathodically are indicated by a minus sign. Where an enzyme encoded more than one locus, the loci were numbered beginning at the cathodic end of the gel. Individuals from at least two populations were loaded onto the same gel to allow direct comparisons to be made. Also, a few individuals from the reference population were usually included in each gel. After scoring, a diagram was made of the gel and kept as a reference. The gels were also fixed for about 30 minutes in a solution consisting of 400ml ethanol, 160ml glacial acetic acid, 80ml glycerol and 320ml water (Smith, 1976), and then wrapped in plastic wrap and kept for possible later reference.

The genotype data for all individuals were entered into a computer, and a series of analyses were performed, mostly using the BIOSYS-1 computer program (release 1.7) of Swofford and Selander (1981). For each sample the mean number of alleles per locus, the percentage of polymorphic loci (P) and the mean heterozygosity per locus (H) were determined. A locus was considered polymorphic if there was more than one allele present in the sample (for sample sizes of less than 50, the 0.99 criterion is not reasonable, Nei, 1987). H was determined by direct count. Deviations of genotype frequencies from expected Hardy-Weinberg proportions were tested for each locus using Fisher's exact test, and also Wright's fixation index, *F*. This index ranges from +1 to -1, with a positive value indicating a deficit of heterozygotes, and a negative value indicating an excess of heterozygotes. When there are only two alleles, the Exact test is the best method (Elston & Forthofer, 1977). When there were more than two alleles, both tests were used. The Exact test can only be used with two alleles, hence the genotypes were pooled into three classes as follows: 1) homozygotes for the most common allele; 2) heterozygotes for the most common allele and any other allele; 3) all other genotypes. However, pooling can obscure real deviations from Hardy-Weinberg proportions, therefore *F* was also tested for significant deviations from zero. This statistic indicates whether or not there is a deficiency or excess of heterozygotes, and is summed over the heterozygote genotypes. No pooling of the genotypes is required. The deviation of *F* from zero was tested using $\chi^2 = NF^2$ and df=1, where N = sample size (Nei, 1987). Contingency table analysis, using the G-test (Sokal & Rohlf, 1981), was used to test for significant allele frequency heterogeneity among populations. Rare alleles were pooled with the common allele, to reduce the number of

cells with low expected frequencies. The G-test was used in preference to the chi-square test as it appears to be less affected by small expected frequencies. Williams' (1976, in Sokal & Rohlf, 1981) correction for G was used because it provides a better approximation to the chi-square distribution. The effect of this correction is to reduce the observed value of G slightly which results in a more conservative test. When sample sizes are large the correction has little effect.

Wright's (1978) hierarchical *F*-statistics were calculated to examine the genetic structuring both within and between populations. Nei's (1978) unbiased distance (*D*) and identity (*I*), as well as the modified Rogers' distance (*D* - Wright, 1978), were calculated for all pairwise comparisons of populations. Nei's identity matrix was clustered using the UPGMA algorithm of Sneath and Sokal (1973). The distance Wagner procedure (Farris, 1972) was used to construct a tree based on the modified Rogers' distance matrix.

Pooling of populations

Of the 38 samples shown in Table 1, six consisted of individuals collected on different dates, and a further six were reduced to two samples by pooling samples taken from separate (but nearby) sites. Therefore, a total of 34 populations were studied.

One of the pooled populations, B-AF, consisted of individuals of an apparently undescribed species collected from several different localities (Table 1). Most of them (15/19) were collected at A-Frame, on three different trips, 4 months apart. This species was very rare, hence only a few individuals were found on each collection. Three individuals were found at Rooiels, 76 km away, and one other at Castle Rock, 4 km away. Although these last four were collected at different sites and on different dates, the data obtained for these four individuals were consistent with those from the A-Frame samples. Another of the pooled populations, CIN-SB, was made up of three populations of *B. cincta* that were collected on the same day but at different sites, but the sites were within 14 km of each other (Table 1).

To ensure as far as possible that there were no significant differences between the individual samples, and therefore that pooling of different collections was permissible, the data were tested for significant differences in allele frequency using a contingency table with the G-test (Sokal & Rohlf, 1981). The probability level of the rejection criteria was adjusted according to Cooper (1968), to compensate for the chance of making a type 1 error when several tests of the same hypothesis are made simultaneously. The overall significance level, summed over the loci was, therefore, 0.05. Most of the polymorphic loci were tested, except in the B-AF population, where there were cells with low expected frequencies. There were no significant allele frequency differences between any of the samples tested, indicating that pooling was acceptable.

RESULTS

Description of loci

In total, 25 enzyme loci were examined, of which three, *LDH*, *ME-1* and *SOD*, were invariant across all 34 populations. A further three, *G6PDH*, *MDH-2* and *ME-3*, were also monomorphic in all populations, but not for the same allele in all populations. A brief description of the polymorphic loci follows. The subunit structure of the enzymes conforms to those found in other molluscs (e.g. Johnson *et al.*, 1977; Grant *et al.*, 1984) unless otherwise specified.

Arginine kinase (*ARK*):

This enzyme showed very strong activity in all samples, with six alleles being detected. Resolution of the bands was good, and heterozygotes showed a two-banded pattern indicative of a monomer, although Murphy *et al.* (1990) say that this enzyme is a dimer.

Aspartate aminotransferase (*AAT*):

Two zones of banding were observed, one cathodic (*AAT-1*) and the other anodic (*AAT-2*), with four and three alleles respectively. Heterozygotes were triple banded, with the centre band darker than the outer two, indicating a dimeric subunit structure. Two buffer systems were used to resolve these loci, and although both loci stained up on both systems, with the same patterns, the resolution of each was better using the respective systems indicated in Table 2.

Diaphorase (*DIA*):

At least two zones of banding were detected for this enzyme. The bands in the lower zone (*DIA-1*), although fainter and not as sharp, could be reliably scored, there being two alleles. Although the two alleles could be differentiated, they were not well separated, and heterozygotes consisted of a blur between the two alleles, so that the subunit structure was not clear. May *et al.*

(1979) describe this enzyme as a monomer in trout. The more anodic zone had much clearer bands, but the pattern was complex, with possibly two overlapping loci, and was not interpretable.

Glucose-6-phosphate isomerase (*GPI*):

A single locus was detected, having eight alleles. Enzyme activity was strong in all samples, and resolution was good. In most samples there were two satellite bands, of decreasing intensity, above the primary band. Heterozygotes were triple banded (as expected), but could be clearly distinguished from the homozygotes with satellite bands.

Glyceraldehyde-3-phosphate dehydrogenase (*GAP*):

This enzyme was polymorphic only in the two populations of *B. catarrhacta*, with two alleles identified. Populations of the remaining species were monomorphic for a third allele. Heterozygotes in the *B. catarrhacta* populations consisted of a smudge between the two homozygote bands and, as such, the subunit structure was not apparent. In mammals (Harris & Hopkinson, 1976), and in cichlids (Van der Bank et *al.*, 1989), a tetrameric subunit structure has been described.

Hexokinase (*HEX*):

Initial screening using the stain for this enzyme, as well as stains for Adenylate kinase (*AK*) and Creatine kinase (*CK*), gave similar banding patterns for all three, irrespective of the buffer system used. The staining ingredients for the three enzymes are very similar and, as Harris and Hopkinson (1976) note, *AK* will stain up when testing for *CK* and *HEX*. There were at least three, possibly four loci. However, most of the banding zones were faint or streaky, making interpretation impossible. The most anodic zone, when stained using the ingredients for *HEX* and buffer system 6, gave good results. Heterozygotes were clearly three-banded, indicating a dimer. This locus is hereafter referred to as *HEX-3*, although it could also be either *AK* or *CK*. Both Harris and Hopkinson (1976), and Murphy et *al.* (1990) note that *AK* is a monomer, and that *CK* is a dimer, although Van der Bank et *al.* (1989), working on cichlids, found that both *AK* and *CK* showed dimer patterns. Murphy et *al.* (1990) regard *HEX* as a monomer, whilst Harris and Hopkinson (1976) find

that of the four loci that have been described in mammals, two are monomers, and the subunit structure of the other two remains uncertain. In a study on barnacles, Hedgecock (1979) found that banding patterns of the heterozygotes were too diffuse to allow accurate subunit determination.

Isocitrate dehydrogenase (*IDH*):

Two loci were observed for this enzyme, and as described for *AAT* above, two buffer systems were used, with each locus having a better resolution using the system indicated in Table 2. The use of NADP in the homogenizing buffer was essential for good resolution of these loci. Without it, many of the samples gave no indication of activity. Three alleles segregated at both loci, and heterozygotes were triple-banded as expected for this dimeric enzyme .

Leucine aminopeptidase (*LAP*):

The products of a single locus with five alleles was detected for this monomeric enzyme. Resolution was good, although the mobilities of the alleles were similar.

Malate dehydrogenase (*MDH*):

Three loci of this dimeric enzyme were detected. The slowest moving zone had three of its four alleles migrating cathodically. The second zone also showed one cathodically migrating band, although there was no confusion between these first two loci. The third locus was relatively very fast moving, and three alleles were found. All three loci showed the presence of two or three satellite bands of decreasing intensity above the primary band.

Mannose-6-phoshate isomerase (*MPI*):

A single zone of activity was seen. The bands, although not sharp, were mostly well separated, and scoring was thus fairly easy. The heterozygotes were clearly double-banded, indicating a monomeric subunit structure. Six alleles were observed.

Octopine dehydrogenase (*ODH*):

One locus with seven alleles was identified for this monomeric enzyme. All of the bands had a single, fainter, satellite band staining up above the main band. This has also been reported for some bivalve species (Beaumont *et al.*, 1980). Some of the samples showed weak activity and could not always be reliably scored, especially amongst the *B. cincta* populations.

Peptidase 1 (*GL*):

This peptidase used the dipeptide glycyl-leucine as the substrate. Two zones of banding were observed, but the less anodic zone was inconsistent and did not appear for all species. The upper zone, although slightly blurred, was scorable with six alleles. The heterozygotes showed the triple-banded pattern of a dimer.

Peptidase 2 (*PHP*):

This peptidase used the dipeptide phenylalanyl-proline as the substrate. One locus was detected with five alleles. As with *GL*, the bands were slightly blurred, but the alleles were clear, and three-banded heterozygotes were observed.

Phosphoglucomutase (*PGM*):

A single locus was detected, with five alleles. Enzyme activity and resolution were good, and double-banded heterozygotes were seen in this monomeric enzyme.

6-Phosphogluconate dehydrogenase (*PGD*):

A single cathodically migrating locus was identified, although two of the nine alleles observed migrated anodically. Resolution was good, and for most samples activity was strong. Alleles were well separated and heterozygotes were triple-banded.

Sorbitol dehydrogenase (*SDH*):

Four alleles in a single locus were observed. Activity was strong but the bands were slightly fuzzy. Two of the bands were not well separated, and it was occasionally difficult to score some of

the samples. Heterozygotes appeared as a solid band between the two homozygote bands. In a study on barnacles, Hedgecock (1979) also found that the subunit structure of this enzyme could not be determined because the banding patterns of the heterozygotes were not sufficiently separated. This enzyme has been described as a tetramer in the American eel (Williams *et al.*, 1973), in cichlids (Van der Bank *et al.*, 1989) and in humans (Harris & Hopkinson, 1976).

Deviations from Hardy-Weinberg

The Hardy-Weinberg law describes the relationship between gene and genotype frequencies. It states that in an infinitely large, randomly mating population, both gene and genotype frequencies will remain constant generation after generation, provided that mating is random and there is no selection operating on the genotypes, no migration of individuals into or out of populations, and no differential mutation between allelic states.

If populations are in Hardy-Weinberg equilibrium at the loci examined, then there should be no difference between the observed genotypes and the expected genotypes which are calculated from the gene frequencies. A significant difference between observed and expected genotypes at a particular locus would indicate that the population is not in Hardy-Weinberg equilibrium, and, therefore that one or more of the conditions necessary for equilibrium have not been met. Cases of selection operating on certain loci have been reported (Koehn *et al.*, 1976; McDonald, 1983; Janson & Ward, 1984), although usually not with a high degree of certainty. The possibility also exists that the loci examined may be linked to other loci that are under selection (Johannesson & Johannesson, 1989). Enzyme loci are not immune to genetic drift or gene flow. Genetic drift may occur if the population size is small, and gene flow can occur if individuals with different allele frequencies migrate into the population.

Testing for Hardy-Weinberg equilibrium not only provides useful information if the populations are not in equilibrium, but is also necessary because a number of statistics, such as

Table 3. Populations and loci showing deviations from Hardy-Weinberg proportions.

Species	Site	Locus	No. of alleles	Exact[1] probability	Wright's *F*
B. cincta	OK	*LAP*	3	0.011	0.521**
	KM	*LAP*	3	0.015	0.348*
	AF	*ODH*	3	0.004	0.461**
	DK	*LAP*	3	0.003	0.379**
B. papyracea	BO	*ODH*	3	0.035	0.289
	AF	*ODH*	2	0.026	0.519**
	RE	*ARK*	2	0.023	0.448*
		AAT-2	2	0.038	0.455*
B. pubescens	CR	*LAP*	3	0.015	0.486**
	AF	*LAP*	3	0.04	0.454*
	PE	*SDH*	2	0.046	0.733*
B. lagenaria	GR	*ODH*	2	0.015	0.624*
	DK	*MPI*	2	0.021	1.0***
	DN	*PGD*	2	0.048	1.0*
B. sp. A	GR	*GL*	3	0.033	0.594**
	BO	*MPI*	2	0.001	0.726***

[1] Fisher's exact test
* $p<0.05$; ** $p<0.01$; *** $p<0.001$

those which estimate heterozygosity, are based on the assumption that the populations are in Hardy-Weinberg equilibrium (Nei & Roychoudhury, 1974).

Of 193 possible Exact tests for deviations of genotype frequencies from expected Hardy-Weinberg proportions (in cases with only two alleles), eight showed significant ($p<0.05$) deviations from Hardy-Weinberg proportions (Table 3). Eight out of 70 cases in which there were more than two alleles showed significant deviations from expectations using the Exact test (Table 3). When Wright's *F* was used, only seven of these indicated significant deviations, and these were all significant using the Exact test, indicating that the pooling necessary for the Exact test did not appear to conceal real deviations from Hardy-Weinberg expectations. In sum, significant differences in 16 out of 263 tests represents just over 6% (Table 3). In all cases, the deviations were due to heterozygote deficits. In a study of the rocky shore whelk *Nucella emarginata*, Palmer et *al.* (1990) reported significant deviations from Hardy-Weinberg expectations at 13% of the loci tested.

Six of the 16 cases of deviations from Hardy-Weinberg proportions occurred in populations that consisted of pooled subpopulations. However, in all six cases, there was a significant deviation (with both the exact test and *F*) from Hardy-Weinberg expectations in one of the subpopulations before they were pooled. This indicates that the pooling of these samples did not result in the observed deviation from Hardy-Weinberg proportions.

Five of the seven species had populations which showed deviations from Hardy-Weinberg proportions: four populations of *B. cincta*, three populations in each of *B. papyracea*, *B. pubescens* and *B. lagenaria*, and both of the *B.* sp. A populations.

Out of the 22 polymorphic loci, deviations from Hardy-Weinberg proportions occurred in eight. More than half of these deviations occurred at two loci. Five populations had deviations at the *LAP* locus, whilst there were four populations with deviations at *ODH*. Two populations showed deviations at *MPI*, and one population each at the *ARK*, *AAT-2*, *GL*, *PGD* and *SDH* loci.

Although the number of deviations from Hardy-Weinberg are not much more than would be expected by chance, this does not imply that the deviations are not real. *LAP* was polymorphic in only 12 of the 34 populations, yet it had significant deviations in five of these. Selection at the *LAP* locus has been reported in the mussel *Mytilus edulis* (Koehn et *al.*, 1976). The possibility of selection acting on the *ODH* and *AAT* loci have been reported in *Littorina saxatilis* (Janson & Ward, 1984 and Johannesson & Johannesson, 1989 respectively) and Wilkins (1977) found that a heat-stable allele of *GPI* appeared to be maintained by selection in *Patella vulgata*.

Most of the differences between observed and expected genotypes, whether significant or not, indicated a deficit of heterozygotes. This has been a common finding in studies of marine molluscs (Berger, 1983; Singh & Green, 1984). Such observed heterozygote deficiencies may reflect real deficiencies in natural populations, or apparent deficiencies due to sampling or experimental errors (Staub et *al.*, 1990). Real deficiencies may be due to inbreeding or natural selection operating against heterozygotes. Apparent deficiencies may be due to scoring biases, presence of null alleles, biased sampling of homozygotes, or the Wahlund effect. The latter results in heterozygote deficits due to the mixing of individuals from two independent gene pools which have different allele frequencies. This mixing of different samples could be due to errors in identification of the individuals, or due to existence of cryptic taxa.

It is possible that some or all of the above mentioned causes may have resulted in the heterozygote deficiencies observed in this study. However, the data are inadequate to determine the precise reasons for the observations. Also, some causes (such as selection), are very difficult to test and confirm. Not withstanding the high proportion of significant heterozygote deficits at the *LAP* locus, the percentage of significant deviations observed (about 6%), is not much more than would be expected by chance alone, and these results are therefore not likely to affect further analysis.

General measures of genetic variability

Only three of the 25 loci were monomorphic across all of the populations tested (*ME-1*, *SOD* and *LDH*). The allele frequencies of the 22 polymorphic loci are given in Appendix A. The mean sample size per locus, mean number of alleles per locus, the percentage of polymorphic loci, and the mean heterozygosity per locus, for each sample, are given in Table 4. The mean number of alleles per locus ranged from 1.1 to 1.8, with the PUB-CR population having the highest value. When averaged over all populations in each of the species, the mean number of alleles per locus ranged from 1.15 for *B. catarrhacta* to 1.60 for *B. pubescens*.

The percentage of polymorphic loci is, to a certain extent, an arbitrary measure, since the definition of what constitutes a polymorphic locus varies between studies. The three criteria that are commonly used are: 1) 0.95 - the frequency of the most common allele does not exceed 0.95, 2) 0.99 - where the frequency of the most common allele does not exceed 0.99, 3) no criterion - where there is more than one allele detected irrespective of the frequencies. The first of these criteria is the most restrictive, in that fewer loci will be defined as being polymorphic. The rationale for using the third criterion in this study, is that although a particular locus may be polymorphic by the 0.99 or even the 0.95 criterion in the actual population due to the presence of rare alleles, when the sample size is less than about 50, these alleles may not be represented in the sample. A further factor that should be considered when making comparisons with other studies is the number of loci examined. The interlocus variance is very large, and studies have shown that about 60 to 70% of loci examined are monomorphic. Consequently, the sampling error when too few loci are used is large (Nei, 1987). However, the 25 loci used in this study are more than adequate to give reliable estimates of the proportion of polymorphic loci, except possibly in the few cases where the sample size was less than about 10 (Nei, 1987; Woodruff & Solem, 1990).

In this study, the percentage of polymorphic loci per sample ranged from 12% in the CAT-DK population, to 56% in the PUB-CR population. The mean for all the populations in each species is also shown in Table 4. *B. catarrhacta* had the lowest value, with only 14% (on average) of

Table 4. Summary of genetic variation in 34 populations of *Burnupena*. Average number of animals scored per locus (n), mean number of alleles per locus (A), percentage of loci polymorphic (P), and mean heterozygosity per locus (H). Standard errors are given in parentheses.

Species	site	n	A	P[1]	H[2]
B. cincta	PT	29.8 (0.2)	1.6 (0.2)	32	0.12 (0.039)
	BB	18 (0.0)	1.6 (0.2)	32	0.113 (0.04)
	OK	29.4 (0.4)	1.5 (0.2)	32	0.116 (0.041)
	KM	27.9 (0.1)	1.4 (0.2)	28	0.108 (0.037)
	CR	25 (0.0)	1.5 (0.1)	36	0.123 (0.04)
	AF	47 (0.4)	1.6 (0.2)	40	0.094 (0.034)
	DK	70.4 (0.3)	1.6 (0.2)	40	0.105 (0.036)
	SB	31 (0.5)	1.6 (0.2)	36	0.132 (0.043)
	MB	47 (1.8)	1.5 (0.2)	28	0.123 (0.044)
	PE	4.4 (0.2)	1.2 (0.1)	20	0.044 (0.026)
	Mean	33	1.51	32.4	0.108
B. papyracea	BB	32.9 (0.1)	1.4 (0.1)	36	0.097 (0.037)
	BO	44.7 (0.1)	1.6 (0.2)	44	0.092 (0.031)
	LL	5 (0.0)	1.3 (0.1)	28	0.12 (0.043)
	CR	42 (0.0)	1.6 (0.2)	40	0.105 (0.035)
	AF	36.1 (0.4)	1.4 (0.1)	36	0.087 (0.032)
	RE	28.4 (0.3)	1.4 (0.1)	32	0.08 (0.029)
	HM	3 (0.0)	1.2 (0.1)	20	0.08 (0.035)
	Mean	27.4	1.41	33.7	0.094
B. pubescens	CR	32.9 (0.1)	1.8 (0.2)	56	0.152 (0.04)
	AF	26.7 (0.6)	1.7 (0.2)	48	0.125 (0.039)
	RE	7 (0.0)	1.5 (0.2)	40	0.12 (0.039)
	PE	9.4 (0.4)	1.4 (0.1)	32	0.069 (0.026)
	Mean	19	1.6	44	0.117
B. lagenaria	GR	19.8 (0.2)	1.2 (0.1)	24	0.062 (0.025)
	BB	15 (0.0)	1.4 (0.1)	32	0.075 (0.026)
	KM	21.5 (0.3)	1.2 (0.1)	16	0.047 (0.027)
	DK	24.8 (0.1)	1.3 (0.1)	28	0.07 (0.04)
	SB	14.8 (0.2)	1.2 (0.1)	20	0.052 (0.024)
	HM	17 (0.0)	1.3 (0.1)	24	0.045 (0.021)
	MB	39.6 (0.2)	1.3 (0.1)	28	0.06 (0.024)
	DN	9.5 (0.4)	1.2 (0.1)	16	0.044 (0.028)
	Mean	20.3	1.26	23.5	0.057
B. sp. A	GR	23 (0.0)	1.3 (0.1)	28	0.052 (0.022)
	BO	44.2 (0.3)	1.4 (0.1)	28	0.051 (0.023)
	Mean	33.6	1.35	28	0.052
B. catarrhacta	KM	19.2 (0.7)	1.2 (0.1)	16	0.063 (0.031)
	DK	26 (1.0)	1.1 (0.1)	12	0.05 (0.029)
	Mean	22.6	1.15	14	0.057
B. sp. B	AF	18.7 (0.3)	1.5 (0.1)	44	0.088 (0.032)

[1]A locus is considered polymorphic if more than one allele was detected.
[2]Direct count

the loci being polymorphic. The next lowest value was for *B. lagenaria*, which at 23.5%, was considerably higher than that for *B. catarrhacta*. Levels of polymorphism for *B.* sp. A, *B. cincta* and *B. papyracea* were 28, 32.4 and 33.7% respectively. For both *B.* sp. B and *B. pubescens*, 44% of the loci were estimated to be polymorphic, which is considerably higher than those estimated for the other species. This increase in the percent of polymorphic loci is also reflected, for the most part, in the mean number of alleles per locus. This would be expected since the higher the mean number of alleles per locus, the greater the number of loci that would be classified as polymorphic.

As with the percentage of polymorphic loci, the estimates of the mean observed heterozygosities can vary a great deal if too few loci are analysed, although the 25 loci examined here are adequate to give reliable estimates of heterozygosity. Several studies have shown that it is more important to examine a large number of loci, due to the large variance between loci, than a large number of individuals, but that increasing the sample size will help to reduce the standard error if a small number of loci are used, and also when heterozygosity values are high (Nei & Roychoudhury, 1974; Nei, 1978). To reduce the standard error of average heterozygosity, Nei (1987) recommends using at least 20 or 30 individuals when about 25 loci are used.

The mean observed heterozygosities, H, (with standard errors), averaged over all loci for each sample are given in Table 4. The values ranged from 0.044 to 0.152, with the CR population of *B. pubescens* having the highest mean heterozygosity. When the heterozygosity levels were averaged over the populations in each of the species, three species, *B. lagenaria*, *B.* sp. A and *B. catarrhacta*, had similar, but relatively low heterozygosities (0.057, 0.052 and 0.057 respectively). The other four species had levels ranging from 0.088 to 0.117. In most of the species, H varied substantially between the populations. The range was greatest in the *B. pubescens* populations (H = 0.069 - 0.152) although the low value of the PE population was responsible for this big range. The *B. cincta* populations also showed a large range (H = 0.044 - 0.133), although again, one population (PE) had a much lower level (less than half the next lowest value).

Table 5. Species-specific alleles, species and populations in which the allele was found, and the number of populations out of the total number collected for that species where the allele was found.

Species-specific allele	Species with allele [1]	population/s having the allele [1]	comments	No. of [2] populations
ARK^{32}	PUB	CR, AF, PE		3/4
ARK^{82}	CAT	KM, DK	both fixed	2/2
ARK^{86}	PAP	BB, BO, LL, CR, AF, RE		6/7
AAT-1^{-61}	B	AF	rare	1/1
AAT-1^{-50}	CAT	KM, DK	both fixed	2/2
AAT-2^{127}	CAT	KM		1/2
G6PDH^{104}	CAT	KM, DK	both fixed	2/2
GPI^{-165}	PUB	CR	rare	1/4
GPI^{-150}	CAT	KM, DK	both fixed	2/2
GPI^{-86}	PAP	AF	rare	1/7
GPI^{200}	PUB	CR, AF, RE		3/4
GPI^{450}	PUB	CR, AF, RE, PE		4/4
GPI^{575}	PUB	PE	rare	1/4
GAP^{-520}	CAT	KM, DK		2/2
GAP^{-450}	CAT	KM, DK		2/2
HEX-3^{92}	LAG	GR		1/8
HEX-3^{97}	CAT	KM, DK		2/2
HEX-3^{101}	CAT	KM,DK		2/2
LAP^{90}	CAT	KM, DK	both fixed	2/2
MDH-1^{-280}	CAT	KM, DK	both fixed	2/2
MDH-1^{-65}	B	AF		1/1
MDH-2^{-67}	CAT	KM, DK	both fixed	2/2
MDH-2^{36}	PUB	CR, AF, RE, PE	all fixed	4/4
MDH-3^{82}	CIN	PT	rare	1/10
MDH-3^{95}	CAT	KM, DK	both fixed	2/2
ME-3^{102}	CAT	KM, DK	both fixed	2/2
MPI^{78}	CAT	KM, DK	both fixed	2/2
ODH^{69}	PAP	BO, CR	rare	2/7
ODH^{85}	PUB	CR, AF, RE	rare	3/4
ODH^{125}	CAT	KM, DK	both fixed	2/2
GL^{91}	PAP	BB	rare	1/7
GL^{119}	CIN	MB		1/10
GL^{129}	CAT	KM, DK	both fixed	2/2
PHP^{81}	A	BO	rare	1/2
PHP^{87}	A	GR, BO		2/2
PHP^{95}	CAT	KM, DK	both fixed	2/2
PGD^{-655}	CAT	KM, DK	both fixed	2/2
PGD^{-490}	LAG	GR, BB, KM, DK, SB, HM, MB, DN		8/8
PGD^{-300}	A	GR, BO		2/2
PGD^{-260}	B	AF		1/1
PGD^{455}	PUB	CR, AF, RE, PE		4/4
PGD^{800}	PUB	PE	rare	1/4
SDH^{77}	CAT	KM, DK	both fixed	2/2
SDH^{118}	PUB	AF, RE		2/4

[1]Species and population abbreviations as in Table 1.

[2]number of populations out of the total collected for each species that have the species-specific allele.

The observed heterozygosities were also considered locus-by-locus for each of the populations in each species (Appendix B). On the whole, the loci were very variable, not only in the number of populations in each species which were polymorphic, but also in the number of species that were polymorphic for the different loci. For example, for *LAP*, most of the populations of *B. cincta* and *B. pubescens* were polymorphic with average heterozygosities of 0.244 and 0.169 respectively. All of the other species were monomorphic at this locus, except for the LAG-BB population, which had a heterozygosity level of 0.333. The loci also had highly variable levels of heterozygosity, again both between populations of the same species, and between the different species. For example, the heterozygosity levels at *IDH-1* for the populations of *B. cincta* ranged from zero to 53 %, and those at *LAP* from zero to 44%.

A few loci were noteworthy (Appendix B). Only one locus, *PGM*, had heterozygous individuals in all of the seven species, and only one of the 34 populations sampled (LAG-KM) was monomorphic at this locus. Furthermore, the heterozygosity levels for this locus were high in all of the species, with the means per species ranging from 0.105 to 0.530. Two other loci, *MPI* and *ARK*, had heterozygous individuals in all of the species with the exception of *B. catarrhacta* (which was monomorphic for these two loci), although the levels varied, as did the number of populations in each species. The heterozygosity levels at the *GPI* locus were also interesting. Apart from the *B. pubescens* populations, only six populations had individuals that were heterozygous (in three species), with levels ranging from 0.024 to 0.063. All four of the *B. pubescens* populations, however, were highly variable at this locus, with the number of heterozygous individuals ranging from 50 to 86%.

Species-specific alleles and diagnostic loci

Two loci, *GPI* and *PGD*, each had six species-specific alleles. Five of the seven species had a species-specific allele for *PGD* in at least one of the populations, but only three species had species-specific alleles for *GPI* (Table 5). The populations of *B. pubescens* had four of the six alleles at the latter locus. Apart from *GPI* and *PGD*, there were another 32 species-specific alleles in 16

Table 6. Populations and species having diagnostic loci.

Locus	species[1]	populations	No. of[2] populations
AAT-1	CAT	KM, DK	2/2
G6PDH	CAT	KM, DK	2/2
GPI	CAT	KM, DK	2/2
GAP	CAT	KM, DK	2/2
HEX-3	CAT	KM, DK	2/2
LAP	CAT	KM, DK	2/2
MDH-1	CAT	KM, DK	2/2
MDH-3	CAT	KM, DK	2/2
ME-3	CAT	KM, DK	2/2
MPI	CAT	KM, DK	2/2
ODH	CAT	KM, DK	2/2
GL	CAT	KM, DK	2/2
PHP	CAT	KM, DK	2/2
SDH	CAT	KM, DK	2/2
ARK	PAP	CR, AF, RE, HM	4/7
ARK	CAT	KM, DK	2/2
MDH-2	PUB	CR, AF, RE, PE	4/4
MDH-2	CAT	KM, DK	2/2
PGD	PAP	BB, BO, LL, CR, AF, RE	6/7
PGD	PUB	AF, RE, PE	3/4
PGD	LAG	GR, BB, KM	3/8
PGD	A	GR, BB	2/2
PGD	CAT	KM, DK	2/2

[1]Species abbreviations as in Table 1.

[2]number of populations out of the total collected for each species that have the species-specific locus.

other loci (Table 5). In total, 44% of the alleles for the polymorphic loci were specific to one species.

B. catarrhacta is very well differentiated, and had 20 species-specific alleles at 18 loci, with the allele fixed in both of the *B. catarrhacta* populations at 15 of them (Table 5). Only one other allele was fixed in all populations of a particular species, namely $MDH\text{-}2^{36}$ in *B. pubescens*. Twelve of the loci had specific alleles for more than one species. The populations of *B. pubescens* had ten species-specific alleles (four of these at the *GPI* locus), with the remaining species having four or fewer. Apart from alleles specific to *B. catarrhacta*, most of the specific alleles occurred in only a few of the populations collected (with a few exceptions, usually in *B. pubescens*), and in quite a few cases the allele occurred at a low frequency.

Ayala (1983) described a method for calculating the probability of assigning an individual to the correct species using alleles as diagnostic characters. Either genotype or allele frequencies can be used to estimate population frequencies, but the latter was used here since this gives a smaller error than using genotype frequencies when sample sizes are not very large. A locus is considered diagnostic if individuals can be assigned to the correct species 99% of the time (Avise, 1975). Using this criterion, and the method described by Ayala (1983), a number of loci were found to be diagnostic, for some of the populations (i.e. individuals of this population could be assigned to the correct species), or for all of the populations tested for the species (Table 6). A population or species does not always need to have species-specific alleles for a locus to be diagnostic, but they must have alleles at a fairly high frequency which are rare in other species.

For example, PGD^{455} is the common allele in all of the *B. papyracea* populations (see Table 7). It was also found at a low frequency, and only as heterozygotes in both populations of *B.* sp. A (0.056 and 0.022 for the A-BO and A-GR populations respectively). It was not found in any of the other populations tested. Assuming that these frequencies are representative of the population, the probability of finding an individual of the latter species which is homozygous for this allele (and therefore not distinguishable from *B. papyracea*) would be $(0.056)^2$ which is 0.003, for the one

Table 7. Details of the number of populations in each species having particular alleles for *PGD*. In some cases the allele frequency is given in brackets .

	species[1]						
allele	CIN	PAP	LAG	PUB	A	CAT	B
800*				1/4 (0.05)			
455*				4/4			
-100	10/10	1/7 (0.167)	5/8	1/4 (0.06)			1/1 (0.816)
-182				3/4			1/1 (0.026)
-260*							1/1 (0.158)
-300*					2/2		
-455		7/7			2/2 (<0.06)		
-490*			8/8				
-655*						2/2	

[1]Species abbreviations as in Table 1.

* species-specific allele

population, and 0.0005 for the other population. That is, there would be a less than 1% chance of finding a PGD^{-455} homozygote in the *B.* sp. A populations, and the probability of making a correct diagnosis of the *B. papyracea* individuals is 0.997 or higher. Therefore *PGD* can be said to be diagnostic for those *B. papyracea* populations where this allele is either fixed, or, if there are other alleles, that these can be distinguished from other populations/species in the same manner as just described. In this particular case (Table 6), *PGD* was diagnostic for six of the seven *B. papyracea* populations. The seventh population, PAP-HM, had the PGD^{-100} allele at a frequency such that individuals could not be assigned to the species with 99% certainty.

As mentioned above, five of the seven species had species-specific alleles for *PGD*. Sixteen of the 34 populations (Table 6) could be diagnosed using this locus, which is much higher than for any other locus. One of the six species-specific *PGD* alleles (Table 7), PGD^{800}, was rare (frequency of 0.05, with one heterozygous individual), and another, PGD^{-260}, although not rare (0.158) was always found in the heterozygous state. The other four species-specific alleles occurred in all of the populations of the species (Table 7), and for *B. lagenaria*, *B.* sp. A and *B. catarrhacta*, was the most abundant allele in all of the populations. Three alleles (PGD^{-100}, PGD^{-182} and PGD^{-455}) were shared by more than one species, of which the latter two were found in only two species (Table 7). In both cases, the allele was the common one for one of the species (PGD^{-182} in *B. pubescens* and PGD^{-455} in *B. papyracea*), and only occurred at a low frequency (< 0.06), and only as heterozygotes, in the other species (PGD^{-182} in *B.* sp. B and PGD^{-455} in *B.* sp. A). The third shared allele, PGD^{-100}, was found in five of the species (Table 7). One of these species, *B. cincta*, was fixed for the allele. In two of the species, *B. papyracea* and *B. pubescens*, this allele was found in one individual in one of the populations, in the heterozygous state, with the alternative allele being the common one for the species. In the fourth species, *B. lagenaria*, the frequency ranged from 0 to 0.44, with a few of the individuals being homozygous. In the fifth species, *B.* sp. B, this allele was the most abundant.

Although the ten *B. cincta* populations were fixed for PGD^{-100}, they could not be unambiguously distinguished by this allele from other species. This is because this allele occurred at a frequency high enough for homozygotes to be found with a greater than a 1% chance in at least

Table 8. Percentages of diagnostic loci distinguishing between pairs of species. Above diagonal: loci distinguishing between all individual populations of one species when compared with all populations of the other species. Below the diagonal: loci distinguishing between the pooled populations of one species and another.

Species[1]	CIN	PAP	PUB	LAG	A	CAT	B
CIN	-	4	20	0	12	72	12
PAP	12	-	16	8	12	72	4
PUB	20	20	-	16	16	72	20
LAG	0	16	20	-	12	68	8
A	12	12	20	16	-	72	4
CAT	72	72	72	72	72	-	76
B	12	12	20	16	4	76	-

[1]CIN = *B. cincta*; PAP = *B. papyracea*; PUB = *B. pubescens*; LAG = *B. lagenaria*; A = *B.* sp. A; CAT = *B. catarrhacta*; B = *B.* sp. B.

one other species. In addition, a further eight populations belonging to *B. lagenaria* (five), *B. papyracea* (HM), *B. pubescens* (CR) and *B.* sp. B could not be diagnosed using this locus because the PGD^{-100} allele occurred at a frequency high enough in each of them, such that homozygous individuals could be found which would therefore not be distinguishable from *B. cincta* individuals.

Apart from *PGD*, sixteen other loci were diagnostic for both populations of *B. catarrhacta* (Table 6). Some populations of *B. papyracea* and *B. pubescens* had two diagnostic loci, and some *B. lagenaria* and both *B.* sp. A populations had one diagnostic locus. In eighteen of the 34 populations, all of the individuals could be assigned to the correct species using a single locus (Table 6). Included in the 16 populations that had no diagnostic loci, were all of the *B. cincta* populations (ten), five of the *B. lagenaria* populations, and the *B.* sp. B population. This does not mean however, that these populations of each species could not be distinguished from other species.

Table 6 shows those loci that are diagnostic for particular populations: that is, all individuals in the population can be assigned to the correct species 99% of the time, when compared to all other populations of all of the other species. When comparisons were made between pairs of species only, the numbers of diagnostic loci increased. Table 8 shows the percentage diagnostic loci between all pairs of species. The results above the diagonal indicate the percentage of loci that can distinguish all populations in the one species from all of the populations in the other species. The results given below the diagonal show the percentage of loci that can distinguish between the species when the data for all of the populations of each species are pooled. In nearly half of the comparisons, there were fewer diagnostic loci when all of the individual populations of each species were compared, since if just one of the populations in one of the species could not be distinguished from any population in the other species for a particular locus, then that locus was not considered diagnostic for the two species compared. A consequence of pooling the data for all of the populations is that, if only one or a few populations of a particular species possess an allele (especially at a fairly low frequency) which results in a particular locus not being diagnostic for individual populations, then it is likely that the overall frequency of that allele in the pooled situation, will be too low to affect the diagnosability of the locus.

As expected, *B. catarrhacta* populations are well differentiated from all other species, with at least 68% of the loci diagnostic for this species. The differentiation between the other species was much less, with only 20% or fewer of the loci being diagnostic. In general, *B. pubescens* was the next most differentiated species, with between 16% and 20% of the loci diagnostic in comparison with other species. Although having no diagnostic loci when compared with all other populations (Table 6), *B.* sp. B was distinguishable from any particular species with at least 4% of the loci diagnostic. There was only one comparison in which there were no diagnostic loci, that between *B. cincta* and *B. lagenaria*. As noted above, none of the ten *B. cincta* and five of the eight *B. lagenaria* populations had diagnostic loci when compared with all other populations.

Although no single locus can be used to distinguish with 99% certainty between *B. cincta* and *B. lagenaria*, it may be possible to do so if a number of loci are considered jointly. For example, using the pooled data for *B. cincta* and *B. lagenaria* and Ayala's (1983) method, the probability of making a wrong diagnosis for *PGD* was 0.019, therefore the probability of a correct diagnosis would be 0.981, almost diagnostic. For *LAP* the probability of making an incorrect diagnosis was 0.032, with a probability of a correct diagnosis being 0.968. Taken individually, neither of these two loci can be used to assign at least 99% of the individuals to the correct species. That is, they are not diagnostic according to Avise's definition (1975). If, however, these two loci are considered jointly, then the probability of making an incorrect assignment would be 0.019*0.032 = 0.0006. Therefore the probability of making a correct assignment would be 0.999. One of the assumptions that Ayala (1983) makes is that both taxa are represented in the sample in equal numbers. In the worst case, where all of the individuals belong to one of the two taxa, then the probability of making an incorrect diagnosis would be twice as large. Even if this were the case, the probability of making a correct assignment of *B. cincta* or *B. lagenaria* using both loci would be 0.998, based on the pooled data for each of the species, and using only two loci.

Electrophoretic detection of incorrectly identified individuals

Once a pattern of the overall enzyme profile of a species has begun to emerge, it is possible to detect individuals previously misidentified (on morphological grounds) prior to electrophoresis. A number of such "mistaken identities" were detected during the study. The new species, referred to as *B.* sp. B, was first discovered in this way from the sample collected at Castle Rock, which was initially thought to consist of *B. papyracea* and *B. pubescens* only. Prior to dissection, one particular shell appeared slightly different from the others, but it was nevertheless identified as *B. pubescens*. The first indication that it was different came from the *PGD* locus. It was a heterozygote (specimen B1 in Table 9a) and carried the common allele for *B. pubescens*, PGD^{-182}, but also PGD^{-100}, the allele fixed in all populations of *B. cincta* and occurring only at low frequency in *B. lagenaria*, *B. papyracea*, and (rarely) in *B. pubescens*. The initial thought was that it was a hybrid between *B. pubescens* and *B. cincta*, as morphological intermediates between these two species do occur (Kilburn & Rippey, 1982). As it turned out, this was the only individual of *B.* sp. B to have the PGD^{-182} allele, which is otherwise found only in *B. pubescens* (Table 7). However, based on the results from other loci (Table 9a) it was clearly not a hybrid between these two species. At two loci (*DIA-1* and *MDH-2*) all populations of *B. pubescens* and *B. cincta* were fixed for different alleles, so a hybrid would be expected to be heterozygous at these loci having one allele from each of the species. The individual was homozygous at both loci, at *DIA-1* for the *B. pubescens* allele, and at *MDH-2* for the *B. cincta* allele. At two other loci where *B. pubescens* and *B. cincta* do not share certain alleles (*MPI* and *PGM*), the results for this individual also did not fit an expected hybrid result. Further, it had a new allele for *MDH-1* ($MDH\text{-}1^{-65}$, which was subsequently only found in this species with a frequency of 0.237), and for *LAP* this specimen was homozygous for an allele not found in either *B. cincta* or *B. pubescens* (LAP^{103}). The enzyme profile of this animal did not fit that of either *B. pubescens* or *B. cincta*, even allowing for the possible rare occurrence of the PGD^{-182} or PGD^{-100} alleles in *B. cincta* or *B. pubescens*. Based on the electrophoretic results, and the fact that it was noted prior to electrophoresis that there were differences in the shell, it seemed probable that this animal belonged to a new species. A second animal (specimen B2 in Table 9a) collected from Rooiels and now known to belong to *B.* sp. B because of its electrophoretic profile,

Table 9a. Alleles present for particular loci for a number of individuals initially misidentified, as well as those present in certain species. Alleles present in a species at a frequency < 0.05 are indicated by a single ✓, those > 0.05 by ✓✓. Alleles present in misidentifed individuals are indicated by a ✓. Thus, a single allele indicates that the individual is homozygous, two alleles indicates a heterozygote.

		Species[1]				Mis-identified individuals[2]							
Locus	Allele	CIN	PUB	PAP	B	B1	B2	RE1	RE2	RE3	RE4	RE5	RE6
ARK	100			✓✓									
	86			✓✓								✓	
	70	✓✓	✓✓	✓	✓✓	✓	✓		✓	✓	✓		✓
	52	✓✓	✓✓	✓✓	✓✓	✓	✓	✓		✓		✓	
	32		✓										
DIA-1	100	✓✓		✓✓	✓								
	94		✓✓	✓✓	✓✓	✓	✓	✓	✓	✓	✓	✓	✓
LAP	109	✓✓	✓✓					✓					
	106	✓✓	✓✓							✓			
	103				✓✓	✓	✓	✓	?	?	?	?	?
	100	✓✓	✓✓	✓✓					?	?	?	?	?
MDH-1	100	✓✓	✓✓	✓✓	✓✓	✓	✓	✓	✓	✓	✓	✓	✓
	-32		✓✓	✓									
	-65				✓✓	✓	✓						
MDH-2	100	✓✓		✓✓	✓✓	✓	✓	✓	✓	✓	✓	✓	✓
	36		✓✓										
MPI	106	✓✓		✓	✓✓								
	100			✓✓	✓✓			✓	?	?		?	
	97	✓✓	✓✓						?	?		?	
	88	✓✓			✓✓	✓	✓			✓	✓	✓	✓
	75	✓	✓✓										
ODH	115	✓	✓	✓✓									
	100	✓✓	✓✓	✓✓	✓✓	✓	✓	✓	✓	✓	✓	✓	✓
	85		✓✓										
	82	✓✓											
	69			✓									
	62	✓✓			✓✓	✓							
PGM	121		✓✓	✓									
	111	✓	✓✓	✓✓	✓✓								
	100	✓✓		✓✓	✓✓	✓	✓	✓	✓	✓	✓	✓	✓
	86	✓✓		✓									
	69	✓✓		✓									
PGD	800		✓										
	455		✓✓										
	-100	✓✓	✓	✓	✓✓	✓	✓	✓	✓	✓	✓	✓	✓
	-182		✓✓		✓	✓							
	-260				✓✓		✓		✓				✓
	-300												
	-455			✓✓									

[1]Species abbreviations as in Table 1.

[2]B1, B2 = B. sp. B; RE1-RE6 = unidentified individuals from Rooiels.

was also initially incorrectly identified. It was first classified as *B. papyracea* on morphological grounds, but the electrophoretic analysis showed that for a number of loci, for example *LAP*, *MDH-1*, *MPI* and *PGD*, the results were not consistent with the enzyme profile for *B. papyracea*. When this specimen was collected, at least 15 other individuals of the new species had been identified, both morphologically and electrophoretically. Despite this, this animal was initially misidentified.

From the sample collected at Rooiels, there were six individuals that were difficult to identify using morphological criteria. They were all covered with a bryozoan making it likely that they belonged either to *B. papyracea*, *B. pubescens* or *B.* sp. B. As established in Chapter 1, four of these specimens were classified as being most similar to *B. pubescens*, and two were classified as *B.* sp. B, using the discriminant function analysis although, as noted, they appeared to be more similar to the latter on the basis of shell ribbing. The general enzyme profiles of all six were similar to that of *B.* sp. B (specimens RE1 to RE6 in Table 9a), although the results for *LAP* were not resolved well enough to be certain whether they all had the LAP^{100} or the LAP^{103} allele. All of the previous individuals of *B.* sp. B were fixed for the latter allele. However, when other locus combinations were considered (for example *MDH-2*, *PGM* and *PGD*), it was concluded that they most probably belonged to the new species. The results from these individuals have not been included in the main analyses.

Apart from the individual belonging to *B.* sp. B in the Castle Rock sample, a further three individuals from this sample were found to be incorrectly identified. Other individuals in the sample were (correctly) morphologically separated into *B. papyracea* and *B. pubescens*. The electrophoretic results revealed that these three individuals, initially classified as *B. papyracea*, were in fact *B. pubescens*. This was clear from their possession of alleles (for a number of loci), which are found in *B. pubescens* populations but not in *B. papyracea* populations (Table 9b).

As noted in Chapter 1, the individuals in the sample collected at Kommetjie (with the exception of most of the *B. catarrhacta*), were particularly difficult to assign to a species. The

Table 9b. Alleles present for particular loci for a number of individuals initially misidentified, as well as those present in certain species. Alleles present in a species at a frequency < 0.05 are indicated by a single ✓, those > 0.05 by ✓✓. Alleles present in misidentifed individuals are indicated by a ✓. Thus, a single allele indicates that the individual is homozygous, two alleles indicates a heterozygote.

		Species[1]		Mis-identified individuals[2]		
Locus	Allele	PAP	PUB	P1	P2	P3
ARK	100	✓✓				
	86	✓✓				
	70	✓	✓✓			✓
	52	✓✓	✓✓	✓	✓	✓
	32		✓			
DIA-1	100	✓✓				
	94	✓✓	✓✓	✓	✓	✓
LAP	109		✓✓			✓
	106		✓✓	✓	✓	✓
	103					
	100	✓✓	✓✓			
MDH-1	100	✓✓	✓✓	✓		
	-32	✓	✓✓	✓	✓	✓
	-65					
MDH-2	100	✓✓				
	36		✓✓	✓	✓	✓
MPI	106	✓				
	100	✓✓				
	97		✓✓	✓		
	88					
	75		✓✓	✓	✓	✓
ODH	115	✓✓	✓	✓		
	100	✓✓	✓✓	✓	✓	✓
	85		✓✓		✓	
	82					
	69	✓				
	62					
PGM	121	✓	✓✓	✓	✓	
	111	✓✓	✓✓		✓	✓
	100	✓✓				
	86	✓				
	69	✓				
PGD	800		✓			
	455		✓✓	✓		✓
	-100	✓	✓			
	-182		✓✓	✓	✓	✓
	-260					
	-300					
	-455	✓✓				

[1]Species abbreviations as in Table 1.

[2]P1 - P3 = *B. pubescens* from Castle Rock.

individuals were divided morphologically into four groups (A - D), two of which were initially identified as *B. cincta* and *B. lagenaria* (groups A and B = populations LIM-KM and LAG-KM respectively). Based on the electrophoretic results for these two groups, two individuals (specimens K1 and K2 in Table 9c) which were initially thought to be *B. cincta* turned out to be *B. lagenaria*. This was clear, based on the presence of PGD^{-490} coupled with the presence of LAP^{109} and the absence of LAP^{106} and LAP^{100} - a combination that is rare in *B. cincta*. These are the only two loci for which the chances of distinguishing between these two species is relatively high.

The identification of the individuals in the other two groups (groups C and D with six and four individuals respectively) was uncertain but they were morphologically separable into two groups, distinct from each other and from groups A and B. The animals were all smaller in size than those in the first two groups. In group C, one of the four individuals tested (specimen K3 in Table 9c) was definitely *B. cincta* since it was homozygous for PGD^{-100}, whereas all of the *B. lagenaria* KM individuals were homozygous for PGD^{-490} at this site. The other three group C individuals (K4 to K6) were probably also *B. cincta*, although no results were obtained for *PGD* for the last three animals. At the *LAP* locus the allele present was LAP^{106}, found in the main LIM-KM population (Table 9c) whereas the main LAG-KM population was fixed for LAP^{109}. Also, at the *MPI* locus, LAG-KM had no MPI^{88} allele, but three of the four group C animals tested had this allele. At *PGM*, all four animals were heterozygotes for PGM^{100} plus PGM^{86} or PGM^{69}, whilst the main LAG-KM population was fixed for PGM^{100}. Thus, with a high degree of confidence, all four group C animals could be assigned to *B. cincta* despite being considered different from it on morphological grounds. Members of group D were initially thought to be *B. catarrhacta*. Two (specimens K7 and K8 in Table 9c) were tested electrophoretically and both proved to be *B. lagenaria*, based on the results from *PGD*, as well as *LAP*, *MPI* and *PGM*.

Table 9c. Alleles present for particular loci for a number of individuals initially misidentified, as well as those present in certain species. Alleles present in a species at a frequency < 0.05 are indicated by a single ✓, those > 0.05 by ✓✓. Alleles present in misidentifed individuals are indicated by a ✓. Thus, a single allele indicates that the individual is homozygous, two alleles indicates a heterozygote.

		Populations[1]			Mis-identified individuals[2]							
Locus	Allele	LIM-KM	LAG-KM	CAT-KM	K1	K2	K3	K4	K5	K6	K7	K8
ARK	82			✓✓								
	70	✓✓					✓	✓	✓	✓	✓	✓
	52	✓✓	✓✓		✓	✓		✓		✓		
	32											
DIA-1	100	✓✓	✓✓		✓	✓	✓	✓	✓	✓	✓	✓
	94			✓✓								
LAP	109	✓✓	✓✓		✓	✓					✓	✓
	106	✓✓					✓	✓	✓	✓		
	100	✓✓					✓					
	90			✓✓								
MPI	106	✓✓	✓									
	97	✓✓	✓✓		✓	✓	✓	✓		✓	✓	✓
	88	✓✓					✓	✓	✓			
	78			✓✓								
ODH	125			✓✓								
	100	✓✓	✓✓			✓	✓	✓	✓	✓	✓	
	85											
	82	✓✓	✓✓		✓	✓	✓			✓	✓	✓
	62	✓										
PGM	100	✓✓	✓✓		✓	✓	✓	✓	✓	✓	✓	✓
	86	✓✓		✓✓					✓	✓		
	69	✓✓		✓✓			✓	✓				
PGD	-100	✓✓					✓					
	-182											
	-490		✓✓		✓	✓					✓	✓
	-655			✓✓								

[1]Population abbreviations as in Table 1.

[2]K1-K2 = *B. lagenaria*; K3-K6 = *B. cincta*?; K7-K8 = *B. lagenaria*.

Geographic variation in allele frequencies

Differentiation in allele frequency between populations of the same species was tested by means of contingency table analysis, using the G-test adjusted using Williams' correction (Sokal & Rohlf, 1981). Not all of the polymorphic loci could be tested, since in some cases the cells had small expected frequencies, and the cells could not always be pooled. In a few such cases, and where only a couple of populations with small sample sizes were involved (CIN-PE, PAP-HM, PAP-LL and LAG-DN), those populations that had the low values were excluded from the analysis, rather than exclude the locus altogether.

Using equations in Sokal and Rohlf (1981), Grant (1989) generated a table of sample sizes required to detect a true difference in allele frequencies for a 2 x 2 contingency table. He concluded that large sample sizes are required to detect small frequency differences. More specifically, small sample sizes are adequate to detect small differences in allele frequency when these frequencies are close to 1.0 or 0.0, but frequencies close to 0.5 require the largest sample sizes. Using this table, it would appear that, based on most of the sample sizes in this study, only frequency differences of about 0.3 or greater would be detected with about 90% certainty. This does not mean, however, that frequency differences of less than 0.3 will not be detected, just that detection may be less certain. It must also be borne in mind that this table was calculated for a 2 x 2 table, and many of the tests performed were larger than this. Where there are more than two classes, the generation of a table of sample sizes is very difficult (Sokal & Rohlf, 1981)

There was a highly significant difference ($p<0.001$) between the populations in each of the species tested (*B.* sp. B was not tested since there was only one population), when summed over all loci (Table 10a). The populations were also different at the majority of the loci. The collection sites were divided into biogeographic regions as previously described, and further analyses were conducted to try and establish which populations, or groups of populations, were responsible for the significant allele frequency differences between different populations of each species.

Table 10a. Contingency table analysis of intra-specific allele frequency heterogeneity for all species. N is the number of populations. (G adjusted using Williams' correction)

	B. cincta			*B. papyracea*			*B. pubescens*			*B. lagenaria*			*B. sp.* A			*B. catarrhacta*		
N	10			7			4			8			2			2		
Locus	G	df	p	G	df	p	G	df	p	G	df	p	G	df	p	G	df	p
ARK	81.08	9	0.001	81.34[2]	8	0.001	31.87	3	0.001	130.27	7	0.001	+		NS	-		
AAT-1	-			+		NS	+		NS	-			-			-		
AAT-2	74.46[1]	8	0.001	43.32[3]	5	0.001	+		NS	*			18.33	1	0.001	77.82	1	0.001
DIA-1	-			5.06	5	NS	-			102.29	7	0.001	-			-		
GPI	+		NS	+	a	NS	44.45	9	0.001	-			-			-		
GAP	-			-			-			-			-			14.12	1	0.001
HEX-3	-			*			+		NS	56.75[4]	6	0.001	+		NS	6.55	1	0.025
IDH-1	84.57[1]	8	0.001	19.75[2]	4	0.001	+		NS	61.92	7	0.001	0.81	1	NS	-		
IDH-2	+		NS	-			+		NS	28.62	7	0.001	-			-		
LAP	376.32[1]	16	0.001	-			58.25	3	0.001	55.86[4]	6	0.001	-			-		
MDH-1	-			+	a	NS	4.49	3	NS	-			-			-		
MDH-3	+		NS	-			-			-			-			-		
MPI	113.80	18	0.001	+		NS	3.00	3	NS	*			5.6	1	0.025	-		
ODH	101.36	18	0.001	8.06[3]	5	NS	+	a	NS	93.89	7	0.001	-			-		
GL	51.51[1]	8	0.001	32.16	6	0.001	-			41.9	7	0.001	60.42	2	0.001	-		
PHP	-			-			+		NS	*			6.72	1	0.01	-		
PGM	46.00	18	0.001	3.84[3]	5	NS	7.41	3	NS	309.27	7	0.001	7.38	1	0.01	31.47	1	0.001
PGD	-			#			56.57	3	0.001	64.74	7	0.001	0.85	1	NS	-		
SDH	70.54[1]	8	0.001	23.99[2]	4	0.001	2.19	3	NS	+	a	NS	-			-		
Total	999.6	111	0.001	217.52	42	0.001	208.23	30	0.001	945.51	68	0.001	100.11	8	0.001	129.96	4	0.001

- \- all populations monomorphic.
- * test not performed - expected frequencies < 1.0.
- \+ populations monomorphic except one/two that have rare alleles
- # populations monomorphic except HM.
- [1] test excludes PE population due to small sample size.
- [2] test excludes HM and LL populations due to small sample size.
- [3] test excludes HM population due to small sample size.
- [4] test excludes DN population due to small sample size.
- a three populations have rare allele.
- b populations monomorphic except BB.
- NS - not significant.

B. cincta

Nine of the 12 polymorphic loci showed significant differences in allele frequency between the populations at individual sites (Table 10a). Of the remaining three polymorphic loci, only one or two populations were polymorphic and in these cases, the other allele only occurred at a low frequency.

Some of the populations of this species were collected in the intertidal zone, whilst others were collected subtidally. There did not, however, appear to be any correlation between differences in allele frequency from different localities and whether the populations were collected inter- or subtidally.

When the populations were divided into their biogeographic regions, the contingency table analyses for each of the three regions indicated significant ($p<0.005$) allele frequency heterogeneity when summed over the loci tested (Table 10b).

The variation between the populations was complex. Not all of the loci that contributed to the overall heterogeneity between the populations showed differences in each of the regions, and those that varied between populations in one region, did not necessarily do so in other regions. In the South Coast region, only two of the five loci that could be tested, showed significant differences in allele frequency. There was a different common allele in the two populations at these loci. The results for the other three loci that indicated non-significant differences between the two populations must be treated with caution. The sample size of the PE population is too small to be sure of detecting a real difference. It is likely that there is a difference between the two populations at the *AAT-2* locus, since the PE population was fixed for $AAT\text{-}2^{100}$, and the frequency of this allele at Mossel Bay (MB) was 0.68.

Table 10b. Contingency table analysis of intra-region allele frequency heterogeneity for *B. cincta*. N is the number of populations. (G adjusted using Williams' correction)

	West Coast N=4			Western Overlap N=4			South Coast N=2		
Locus	G	df	p	G	df	p	G	df	p
ARK	26.38	3	0.001	4.52	3	NS	-		
AAT-2	11.40	3	0.01	31.41	3	0.001	2.01	1	NS
IDH-1	-			10.21	3	0.025	0.59	1	NS
LAP	28.29	6	0.001	4.73	3	NS	-		
MPI	40.91	6	0.001	21.06	6	0.005	0.93	1	NS
ODH	7.36	6	NS	19.85	6	0.005	12.39	2	0.005
GL	4.25	3	NS	9.47	3	0.025	*		
PGM	8.18	6	NS	12.47	6	NS	7.68	2	0.025
SDH	21.12	3	0.001	26.73	3	0.001	*		
Total	147.89	36	0.001	140.45	36	0.001	23.60	7	0.005

- all populations monomorphic.

* test not performed - expected frequencies < 1.0.

NS - not significant.

The Western Overlap populations showed significant differences at six out of nine loci, and significant differences were detected at five out of eight loci between the West Coast populations. Where allele frequency differences between the populations within a region were detected, the population/s that were responsible for the heterogeneity, varied for the different loci. However, two populations were responsible for the differentiation more often than other populations. In the Western Overlap region, the Sparks Bay (SB) population appeared to be the cause of the variation at two of the loci (*AAT-2* and *ODH*). This population is separated from its nearest neighbour by about 80km of coastline. The other three populations in this region are separated by about 17km. In the West Coast region, the Paternoster (PT) population was responsible for the differentiation in the area at two loci (*MPI* and *ARK*). Again, it is further away from the other three populations (approx. 140km from its nearest neighbour) than these populations are separated from each other (about 60km apart).

The allele frequencies generally varied haphazardly, both within regions as well as between regions (Fig. 1a). Only the following alleles showed geographic clines and, even then, mostly within a region: MPI^{88}, LAP^{106} and GL^{115} in the West Coast region; SDH^{100} in the Western Overlap region. One allele, ODH^{100}, showed a cline extending over more than one region, and involved six of the 10 populations. This allele decreased in frequency from the Dalebrook (DK) population in the Western Overlap region to Blouberg (BB) on the West Coast.

At five loci, not all of the populations shared the same common allele (Fig. 1a). At the *MPI* locus, the common allele in the MB population was MPI^{88}, whereas, in all of the other populations, the common allele was MPI^{97}. The PT population had a different common allele at the *ARK* locus, ARK^{70}, the common allele in the other populations being ARK^{52}. There were three common alleles for *PGM*: PGM^{69} at PE; PGM^{86} at DK and Oudekraal (OK); and PGM^{100} in the remaining seven populations. For *ODH*, there were two common alleles, ODH^{100} and ODH^{82}. Except for the MB and SB populations, the common allele alternated between the different regions. This pattern was more obvious at the *LAP* locus. LAP^{106} was common in all populations in the South Coast and West Coast regions (it was fixed in the former), whilst LAP^{100} was the common allele in all of the Western

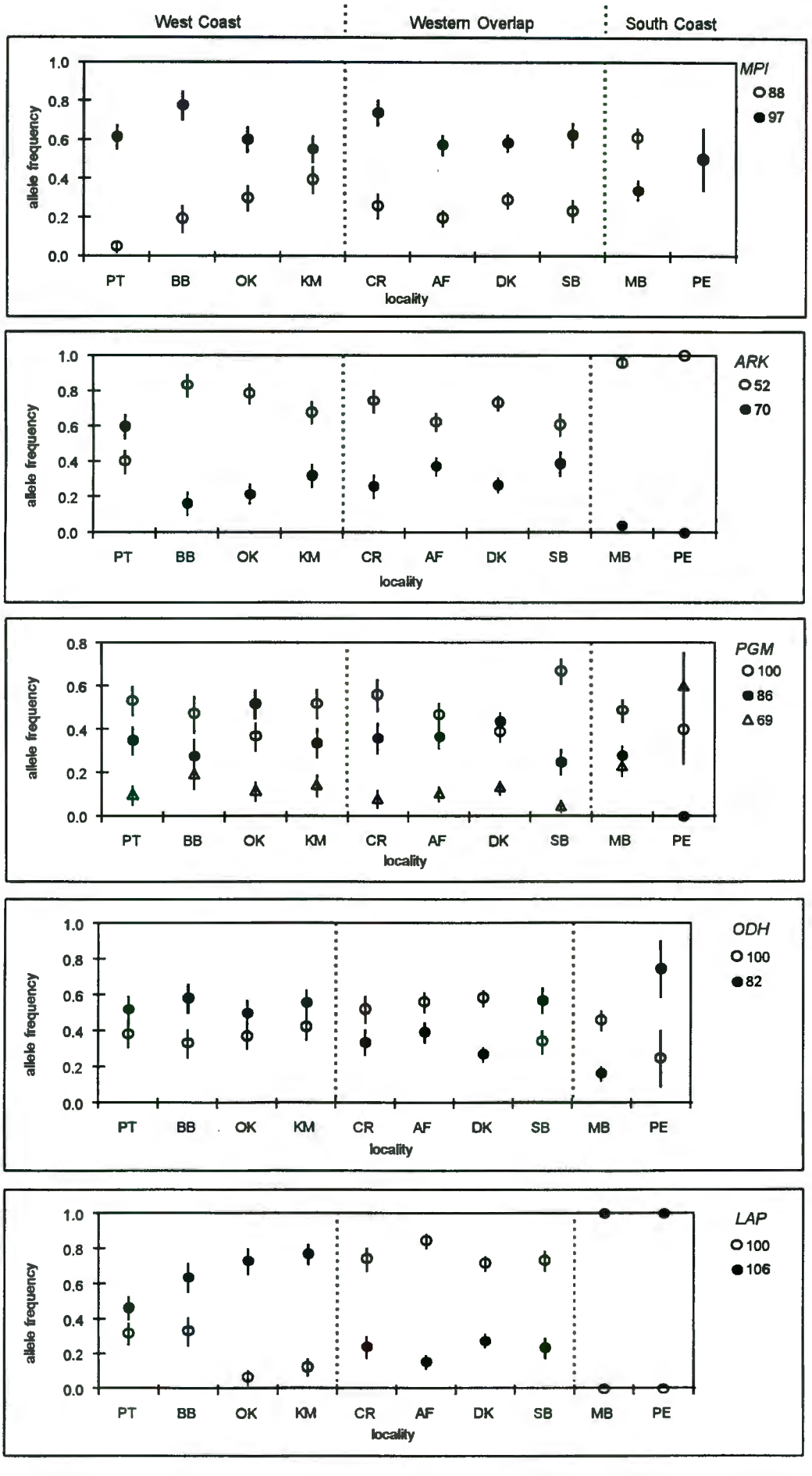


Figure 1a. Frequencies of the most common alleles for five loci for *B. cincta*. Vertical bars represent the standard error $[p(1-p)/2n]^{-1/2}$, where p = allele frequency and n = sample size (Ferguson, 1980). The dashed lines separate the regions.

Overlap populations. The frequency difference between these latter two alleles was fairly large in most of the populations.

B. papyracea

There were 14 loci where at least one of the *B. papyracea* populations was polymorphic (Table 10a). At four of these loci (*AAT-1*, *GPI*, *MDH-1* and *MPI*) a few of the populations were polymorphic, but the other allele/s had very low frequencies. At the *PGD* locus there was only one polymorphic population, Hermanus (HM), but here the other allele, PGD^{-100}, had a frequency of 0.167. The significance of this is questionable, as only three individuals were collected at this site, one heterozygous animal being the only individual to possess PGD^{-100}. At another locus, *HEX-3*, there were three polymorphic populations, but, due to there being cells with low expected frequencies, this locus was not tested between sites. Only five of the polymorphic loci showed significant allele frequency heterogeneity between the populations (Table 10a). These loci were analysed by region, and the results are given, together with those for *HEX-3*, in Table 10c. Significant allele frequency heterogeneity was detected between the four populations in the Western Overlap region at three of the four loci tested. Only at the *GL* locus was no difference detected. At one locus (*ARK*), the HM population was different to the other three populations, and at the other two loci, *IDH-1* and *AAT-2*, the Rooiels (RE) population was different. There was no significant difference between any of the *B. papyracea* populations at the latter two loci when the RE population was excluded from the analysis. The West Coast populations were more homogeneous than the Western Overlap populations. Only one of the five loci tested showed significantly different allele frequencies.

With the single exception of the HM population with respect to *ARK*, the significant differences between all of the populations at three loci, namely, *SDH*, *ARK* and *GL*, were due to differentiation between the two regions. At the latter two loci, the populations within one of the regions had a different common allele from the populations in the other region (Fig. 1b). GL^{100} was

Table 10c. Contingency table analysis of intra-region allele frequency heterogeneity for *B. papyracea*. N is the number of populations. (G adjusted using Williams' correction)

	West Coast N=3			Western Overlap N=4		
Locus	G	df	p	G	df	p
ARK	6.22	4	NS	9.80	3	0.025
AAT-2	0.31[1]	1	NS	28.23	3	0.001
HEX-3	4.41[1]	1	0.05	-		
IDH-1	-			7.36[2]	2	0.05
GL	2.64	2	NS	4.79	3	NS
SDH	2.03[1]	1	NS	-		
Total	15.61	9	NS	50.18	11	0.001

- all populations monomorphic.

[1] = test excludes LL population due to small sample size.

[2] = test excludes HM population due to small sample size.

NS - not significant.

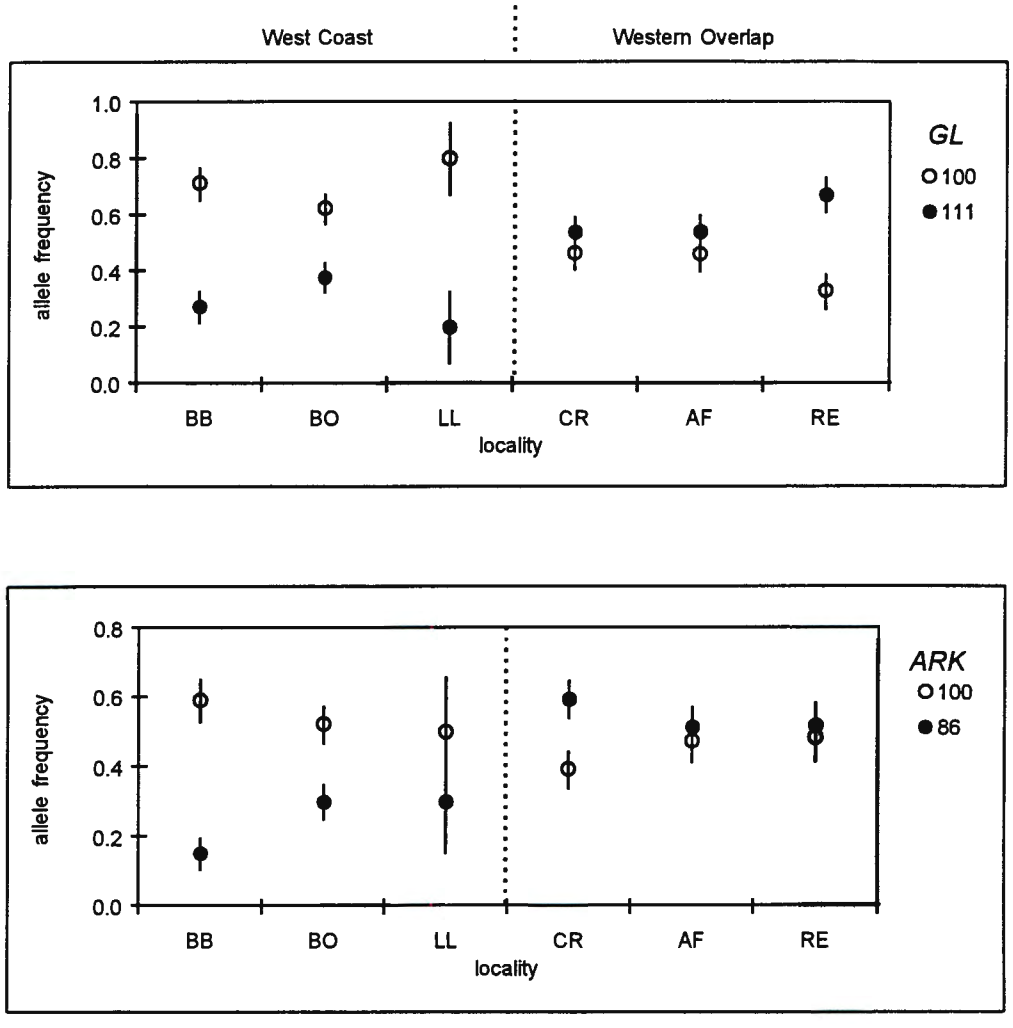


Figure 1b. Frequencies of the most common alleles for two loci for *B. papyracea*. Standard error bars as in Fig. 1a.

Table 10d. Contingency table analysis of intra-region allele frequency heterogeneity for *B. pubescens*. N is the number of populations. (G adjusted using Williams' correction)

	Western Overlap N=3		
Locus	G	df	p
ARK	1.26	2	NS
GPI	8.30	6	NS
LAP	0.05	2	NS
PGD	6.09	2	0.05
Total	15.70	12	NS

NS - not significant.

the common allele in the West Coast populations, whilst GL^{111} was the common allele in the four Western Overlap populations. The situation at the *ARK* locus was similar, with the West Coast populations having ARK^{100} as the most common allele, and the Western Overlap populations, excluding HM, with ARK^{86} the most common.

B. pubescens

The populations of *B. pubescens* were polymorphic at 15 loci (Table 10a). No allele frequency heterogeneity was detected between the four populations at 11 of these loci. At seven of these 11 loci, one or two of the populations were polymorphic but the frequency of the second allele in each case was very low. At the other four loci, all four populations were polymorphic. With some of the small sample sizes, however, it is possible that not all of the differences were detected.

Four loci showed significant differences in allele frequency between the populations. These loci were re-analysed using the Western Overlap populations only (Table 10d). Only at the *PGD* locus was there a difference detected between these three populations, the Castle Rock (CR) population being responsible. However, at all four of these loci, the Port Elizabeth population had a different common allele to the three Western Overlap populations (Fig. 1c), which was the major reason for the significant differentiation seen in Table 10a.

B. lagenaria

Amongst the populations of *B. lagenaria*, there were 14 polymorphic loci (Table 10a). Of the 10 that could be tested, all indicated that there was significant allele frequency heterogeneity between the eight populations. The Durban (DN) population was excluded from the analysis for two of these loci (*LAP* and *HEX-3*). Of the four loci that could not be tested at this level, three (*AAT-2*,

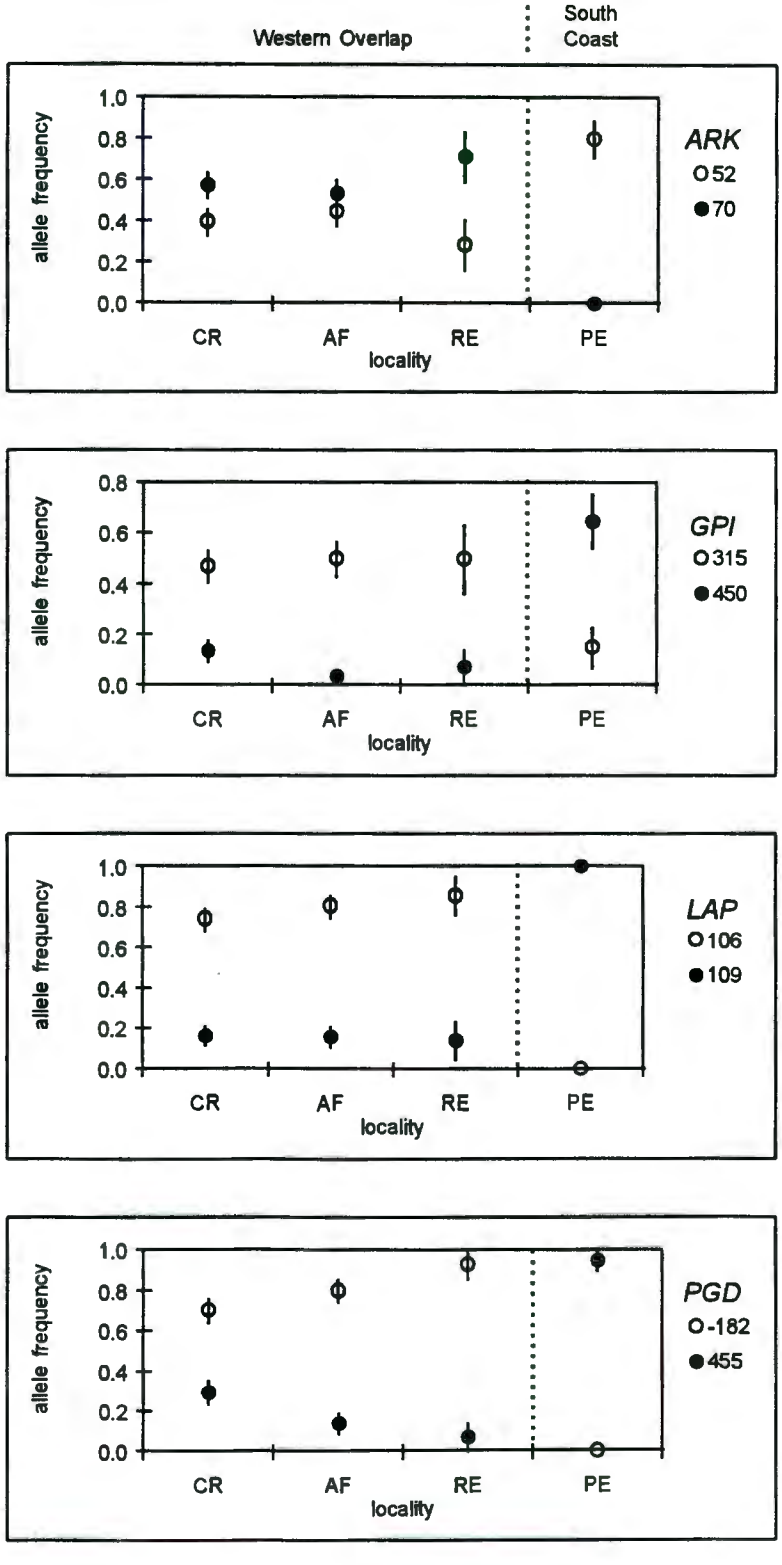


Figure 1c. Frequencies of the most common alleles for four loci for *B. pubescens*. Standard error bars as in Fig. 1a.

MPI and *PHP*) had cells with low expected frequencies as a result of most of the populations being monomorphic, and the other (*SDH*) was polymorphic in only a few populations, but with the other allele present at a low frequency. All but the latter locus were tested for heterogeneity of alleles between the populations within the Western Overlap and West Coast regions (Table 10e).

The three loci not tested over all populations (*AAT-2*, *MPI* and *PHP*), all showed significant differences between some of the populations. At *MPI*, the populations within the two regions were virtually monomorphic. However, the DN and Mossel Bay (MB) populations were different from the other populations, as well as from each other (having different alternative alleles). *AAT-2* showed a significant difference between the Western Overlap populations, and at *PHP*, a significant difference was detected between the West Coast populations.

Among the populations in the Western Overlap region, there were nine polymorphic loci, four of which exhibited significant differences between the populations. The population responsible for this difference at two of these loci was Sparks Bay (SB). In the West Coast area, there were ten polymorphic loci, and eight of these had significant allele frequency heterogeneity. Half of these differences were due to the Blouberg (BB) population being significantly different to the other two populations in this region. At six of the polymorphic loci (*ARK*, *AAT-2*, *HEX-3*, *IDH-1*, *LAP* and *PHP*), only one out of all eight populations sampled around the whole coast was responsible for the significant difference detected.

Not all of the eight populations shared the same common allele at five of the loci (Fig. 1d). At one of these loci, *ARK*, all of the populations were monomorphic for ARK^{52}, or essentially so (one population, BB, had a rare alternative allele), but the DN population was fixed for ARK^{70}. This locus was diagnostic (according to the 99% criterion - Ayala, 1983) for the DN population within this species. Similarly at *IDH-1*, the DN population had a different common allele from the remaining populations. The Groen River population on the West Coast had $DIA\text{-}1^{94}$ as its common allele, although the S.E. estimates of the frequency of this and that of $DIA\text{-}1^{100}$ do overlap. The closest site to GR that was sampled, BB, also showed the trend towards a change in the common

Table 10e. Contingency table analysis of intra-region allele frequency heterogeneity for *B. lagenaria*. N is the number of populations. (G adjusted using Williams' correction)

	West Coast N=3			Western Overlap N=3		
Locus	G	df	p	G	df	p
ARK	+		NS	-		
AAT-2	-			13.52	2	0.005
DIA-1	39.59	2	0.001	-		
HEX-3	32.41[1]	2	0.001	+		NS
IDH-1	-			-		
IDH-2	14.78	2	0.001	+		NS
LAP	36.00[1]	2	0.001	-		
MPI	+		NS	+		NS
ODH	8.92	2	0.025	30.42	2	0.001
GL	15.26	2	0.001	5.64	2	NS
PHP	13.81[1]	2	0.005	+		NS
PGM	12.67	2	0.005	24.25	2	0.001
PGD	-			9.14	2	0.025
Total	173.44	16	0.001	82.97	10	0.001

- all populations monomorphic.
+ populations monomorphic except one that has a rare allele.
[1] = only one population polymorphic.
NS - not significant.

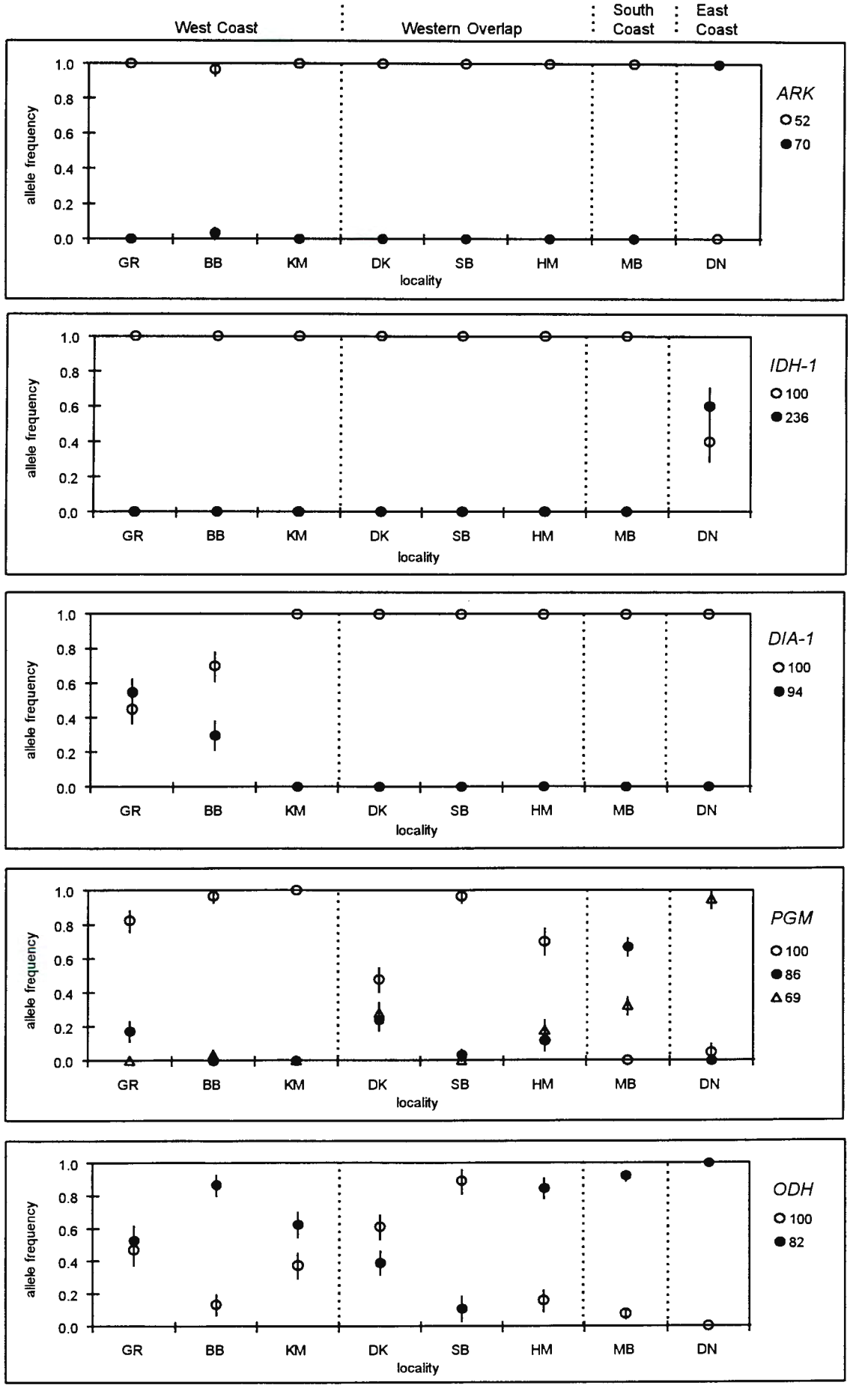


Figure 1d. Frequencies of the most common alleles for five loci for *B. lagenaria*. Standard error bars as in Fig. 1a.

allele. Like the populations of *B. cincta*, there were three common alleles in the *B. lagenaria* populations at the *PGM* locus. The common allele at DN was PGM^{69}, that at MB was PGM^{86}, and for the rest of the populations, PGM^{100}. It is worth noting, that only the most easterly population collected, for both *B. cincta* and *B. lagenaria*, had as it's common allele PGM^{69}. There were two common alleles for *ODH*, ODH^{100} and ODH^{82}. As with *B. cincta*, ODH^{82} was common in the West Coast populations, whereas ODH^{100} was common in most of the Western Overlap populations, and ODH^{82} again common in the most easterly populations.

***B.* sp. A**

There were nine loci at which at least one of the two populations of *B.* sp. A was polymorphic (Table 10a), but at only two of these, were both populations polymorphic. Tests for allele frequency heterogeneity between the two populations at seven of the loci detected significant differences at five of them. Only *PGD* and *IDH-1* (the latter with both populations polymorphic) showed no differences. At only one locus, *AAT-2*, did the populations have a different common allele, and although both populations are in the West Coast region, they are well separated geographically (450km).

B. catarrhacta

There were only four loci that were polymorphic in the two *B. catarrhacta* populations, and at all four, significant differences were detected between the two populations (Table 10a). The populations, one collected in the Western Overlap region and the other on the West Coast, had different common alleles at all of these loci. One of the loci, *GAP*, was polymorphic only in this species. All of the populations of the other species were fixed for GAP^{100}, this allele not being found in the *B. catarrhacta* populations.

Analysis of genetic structure using F-statistics

Wright's (1978) hierarchical *F*-statistics were calculated to examine the genetic structuring (a) within and between populations, and (b) within and between species. This analysis determines the relative amounts of differentiation at different hierarchical levels. Initially, each species was analysed separately. In each of these analyses, the populations (P) were grouped by region (R - see Table 1 for regions), except for *B.* sp. A and *B. catarrhacta*, as only two populations were sampled for each of these species. Also, no analysis was performed on *B.* sp. B species since there was only one population. Finally, the genus as a whole was analysed, all of the populations belonging to the same species (S) being grouped together.

The following abbreviations and *F*-statistics have been used:

1) H_T is the total genetic variance (or total gene diversity) across the populations in the analysis.

2) F_{PT} is that proportion of the total genetic variance attributable to differentiation between all of the populations. The T represents the total population, that is all of the populations in the analysis. This statistic is equivalent to the F_{ST} seen in many studies. In Wright's (1978) terminology, he divides the total population into subpopulations (S), rather than populations. P has been used here, since S is used to represent species (see below).

3) F_{RT} is that proportion of the total genetic variance attributable to differences between regions.

4) F_{PR} is that proportion of the total genetic variance attributable to differences among populations within regions.

5) $1\text{-}F_{PT}$ is that proportion of the total genetic variation attributable to differentiation between individuals within populations.

6) F_{PS} is that proportion of the total genetic variance attributable to differences among populations within species.

7) F_{ST} is that proportion of the total genetic variance attributable to differences between species.

Table 11a. Hierarchical analysis of genetic differentiation between populations of *B. cincta* by Wright's *F*-statistics.

Locus	H_T	F_{PT}	F_{RT}	F_{PR}	1-F_{PT}
ARK	0.387	0.127 *	0.048	0.083	0.873
AAT-2	0.225	0.098	-0.041	0.133	0.902
GPI	0.010	0.008	0.002	0.006	0.992
IDH-1	0.140	0.125 *	0.124	0.001	0.875
IDH-2	0.001	-0.001	-0.001	0.001	1.000
LAP	0.542	0.364 ***	0.336	0.043	0.636
MDH-3	0.003	-0.001	-0.003	0.001	1.000
MPI	0.548	0.063	0.029	0.035	0.937
ODH	0.594	0.060	-0.009	0.069	0.940
GL	0.264	0.069	0.041	0.029	0.931
PGM	0.635	0.049	0.023	0.027	0.951
SDH	0.172	0.104	-0.030	0.131	0.896
over all loci		0.120 *	0.068	0.056	0.880

H_T = total limiting variance (total gene diversity) across populations.

F_{PT} = proportion of total genetic variance attributable to population differentiation.

F_{RT} = proportion of total genetic variance attributable to differences between regions.

F_{PR} = proportion of total genetic variance attributable to among-population within-region variation.

1-F_{PT} = proportion of total genetic variation attributable to differentiation within populations.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

All of the above values are calculated for each locus, as well as over all loci. Only polymorphic loci are used for the calculation of *F*-statistics. An F_{PT} value of zero indicates that, at that locus, there was no differentiation between the populations, and therefore any variation that there may be at that locus (*i*.e. a locus that is not monomorphic), will be due to differences between the individuals within the populations (*i*.e. 1-F_{PT}). The deviation of F_{PT} from zero can be tested using the same formula as that used for Wright's fixation index, that is, $\chi^2 = NF^2_{PT}$ and df = 1, and where N is the total number of individuals in all of the populations tested.

The results of the analyses (Table 11a-g) are discussed below, firstly for each species, and then for the genus as a whole.

B. cincta

The amount of genetic differentiation between the populations of *B. cincta* (F_{PT}), between the regions (F_{RT}), and among the populations within regions (F_{PR}), for the 12 polymorphic loci, is given in Table 11a. The total expected heterozygosity across all of the populations is also shown (H_T). The loci vary considerably in the proportion of heterozygotes, with the values ranging from 0.001 for *IDH-2* (almost monomorphic - one individual in one of the populations was a heterozygote), to 0.635 for *PGM*. Over all of the loci, the within-population differentiation (1-F_{PT}) accounts for about 88% of the total genetic variation, leaving about 12% of the total variation attributable to differentiation between the populations (Fig. 2a). The latter, which is significant ($p < 0.05$) is due, in almost equal amounts, to the differentiation between regions (6.8%), and to the differentiation between populations within regions (5.6%).

The loci did not contribute equally towards the amount of differentiation between the populations. The F_{PT} values ranged from virtually zero (the negative values are as a result of assumptions made for computational purposes - Swofford & Selander, 1981) for three loci, *GPI*, *IDH-2* and *MDH-3*, which were almost monomorphic (four populations had a rare allele), to 0.364

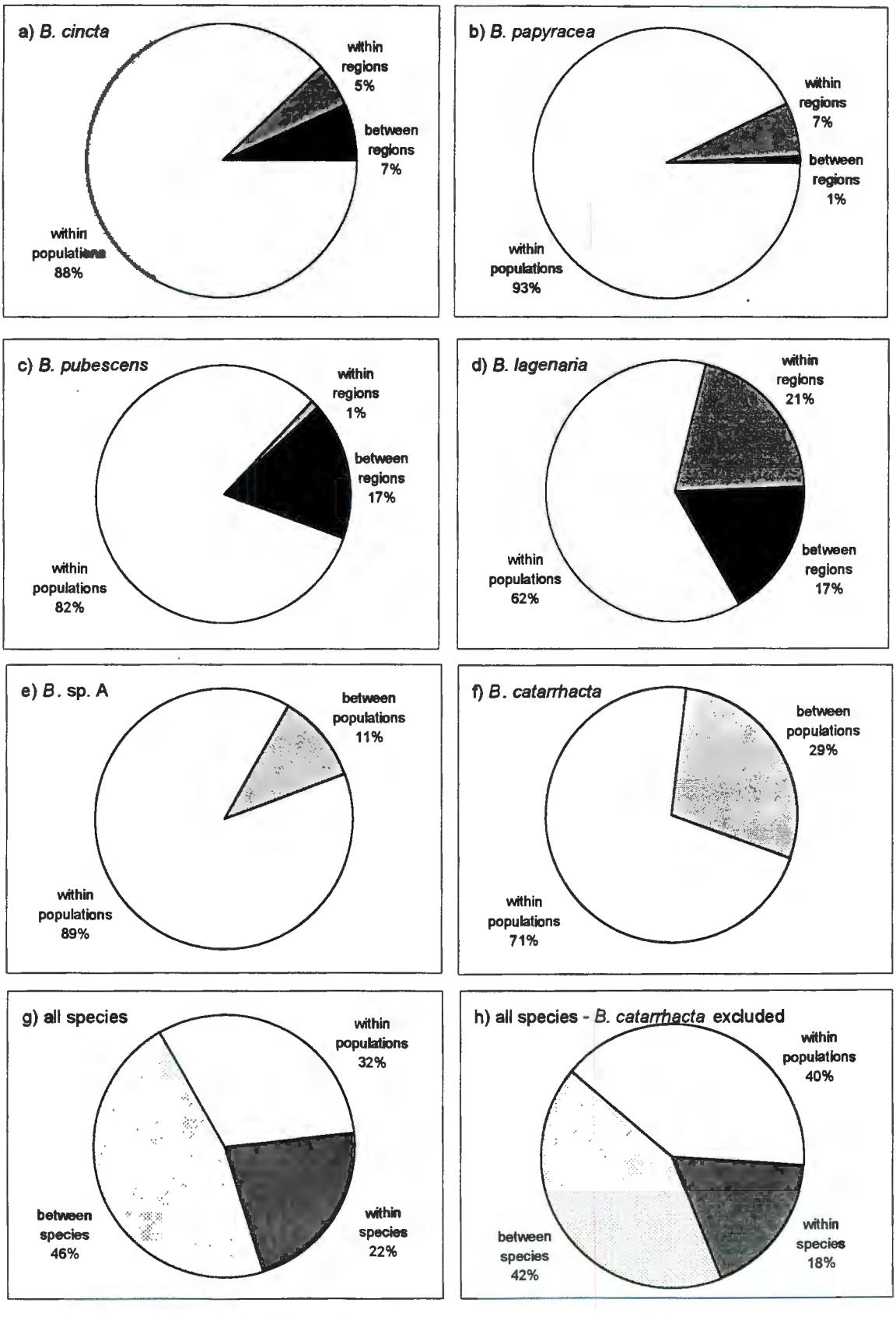


Figure 2. Pie diagrams showing the proportion of genetic variation at different hierarchical levels, using Wright's *F*-statistics, for each species separately (a-f), and all species together (g-h).

for *LAP*. This was nearly three times greater than the next highest value. Of the variation in *LAP* between populations (F_{PT} = 0.364), about 90% was due to the differentiation between regions (F_{RT} = 0.336), with only about 10% due to differences among the populations within a region (F_{PR} = 0.043). This can clearly be seen in Fig. 1a. Two other loci had F_{PT} values higher than the average over all loci, these being *ARK* and *IDH-1* (F_{PT} =0.127 and 0.125 respectively). *IDH-1* showed the same trend as *LAP*, with most of the differentiation attributable to variation between regions (F_{RT} =0.124), with almost no differentiation between populations within regions. The opposite was found at the *ARK* locus, where the differences between regions accounted for about one third of the differentiation (F_{RT}=0.048), whilst the between-population within-region differentiation was two thirds of the total population variance (F_{PR}=0.083).

Considering all of the loci, there was no clear trend showing where most of the differentiation occurred. In about a third of the loci, the differentiation between regions accounted for more of the total variation, than did differentiation between populations within regions. The other two thirds of the loci showed the opposite trend. However, in some of the loci, the difference between the F_{RT} and F_{PR} values was relatively small.

There was no pattern between the total expected heterozygosity (except when the heterozygosity was almost zero), and the proportion of the total genetic variance attributable to population differentiation (F_{PT}). For example *LAP* and *MPI* had similar levels of heterozygosity (H_T=0.542 and 0.548 respectively), yet, *LAP*, as already mentioned, shows significant differentiation among populations (36%), whilst *MPI* showed very little differentiation among populations (6.3%), with most of the variation due to variation within the populations (1-F_{PT})

B. papyracea

The genetic structure within and between the populations of *B. papyracea* was similar to that found in *B. cincta*, although the level of differentiation between its populations was lower

Table 11b. Hierarchical analysis of genetic differentiation between populations of *B. papyracea* by Wright's *F*-statistics.

Locus	H_T	F_{PT}	F_{RT}	F_{PR}	1-F_{PT}
ARK	0.557	0.131	-0.020	0.148	0.869
AAT-1	0.004	-0.001	-0.001	0.000	1.000
AAT-2	0.255	0.160 *	-0.058	0.206	0.840
DIA-1	0.456	-0.008	-0.012	0.005	1.000
GPI	0.012	-0.003	-0.003	0.000	1.000
HEX-3	0.077	0.059	0.009	0.050	0.941
IDH-1	0.080	0.067	0.000	0.067	0.933
MDH-1	0.044	0.028	-0.010	0.038	0.972
MPI	0.013	0.007	-0.008	0.015	0.993
ODH	0.278	-0.007	-0.021	0.013	1.000
GL	0.502	0.137	0.109	0.032	0.859
PGM	0.285	0.004	0.004	0.000	0.996
PGD	0.047	0.004	-0.033	0.036	0.996
SDH	0.056	0.074	0.021	0.055	0.926
over all loci		0.073	0.007	0.066	0.927

For key to abbreviations see Table 11a.

(Table 11b). Fourteen loci were polymorphic, with levels of total heterozygosity ranging from 0.004 for *AAT-1*, to 0.557 for *ARK*. The variability within populations (over all loci) accounted for over 92% of the total genetic variance (Fig. 2b). The variability between populations accounted for the other approximately 7% of the total genetic variability, which is not significant. Unlike the *B. cincta* populations, only a small portion (less than 10%) of the differentiation between populations was attributable to differences between regions (F_{RT}=0.007), over 90% being due to differences between populations within regions (F_{PR}=0.066).

The contribution to the differentiation between populations varied for the different loci. Differentiation between populations at the *AAT-2* locus accounted for 16% of the total variability at this locus. This was the only locus at which significant differentiation ($p<0.05$) was detected between the populations. All of this variation was due to differentiation between populations within regions, rather than to differences between regions. An examination of the allele frequency data (Appendix A) shows that this between-population within-region differentiation was solely as a result of the Rooiels (RE) population which had markedly different allele frequencies from those of the other populations. Two other loci, *ARK* and *GL*, showed a large, but not significant, differentiation between all populations (F_{PT} = 0.131 and 0.137 respectively). All of the variation at the former locus was due to differentiation between populations within regions (F_{PR} = 0.148), similar to the situation for *AAT-2*, but in this case due to the fact that the Hermanus (HM) population was fixed for a different allele from the other populations. If the HM population is excluded from the analyses, the F_{PT} for *ARK* drops from 0.131 to 0.052, and the differentiation between all populations changes from being due to within-region differences, to differences between regions (F_{PR} = 0.000, F_{RT} = 0.051). At the *GL* locus, most of the variation was due to differentiation between the regions (F_{RT} = 0.109), as can be seen in Fig. 1b. Most of the loci showed greater differentiation between populations within regions, as reflected in the totals over all loci. Only *GL* had relatively high levels of differentiation between regions. The other loci had F_{RT} values of about 2% or lower.

Of the 14 loci that were polymorphic in *B. papyracea*, nine were also polymorphic in *B. cincta*. When populations of *B. cincta* and *B. papyracea* are compared, the loci again show no

Table 11c. Hierarchical analysis of genetic differentiation between populations of *B. pubescens* by Wright's *F*-statistics.

Locus	H_T	F_{PT}	F_{RT}	F_{PR}	1-F_{PT}
ARK	0.556	0.189	0.183	0.007	0.811
AAT-1	0.015	0.008	-0.018	0.025	0.992
AAT-2	0.015	0.008	-0.018	0.025	0.992
GPI	0.723	0.123	0.118	0.005	0.877
HEX-3	0.031	0.007	-0.011	0.018	0.993
IDH-1	0.011	-0.005	-0.013	0.008	1.000
IDH-2	0.060	-0.009	-0.009	0.000	1.000
LAP	0.503	0.503 ***	0.499	0.006	0.497
MDH-1	0.309	0.039	0.039	0.000	0.961
MPI	0.456	0.031	0.031	0.000	0.969
ODH	0.105	0.018	0.018	0.000	0.982
PHP	0.047	-0.005	-0.005	0.000	1.000
PGM	0.286	0.029	0.012	0.017	0.971
PGD	0.497	0.494 ***	0.451	0.077	0.506
SDH	0.363	0.000	-0.008	0.008	1.000
over all loci		0.183	0.173	0.012	0.817

For key to abbreviations see Table 11a.

consistent patterns. For example, *LAP*, which showed a substantial differentiation among *B. cincta* populations (36%), was monomorphic in all populations of *B. papyracea*. *ARK*, on the other hand, showed a relatively high level of between-population variability in both species, with most of the variability due to differentiation between populations within regions in both cases.

B. pubescens

The populations of *B. pubescens* were polymorphic at fifteen loci. The levels of heterozygosity ranged from 0.011 for *IDH-1*, to 0.723 for *GPI* (Table 11c). The latter level was the highest reached for any locus in any of the species.

Of the total genetic variance, about 82% was attributable to differentiation within the populations (over all the loci). This was lower than the levels of within-population variance for *B. cincta* and *B. papyracea*. The remaining 18% of the total genetic variance, due to differences between populations, was almost entirely due to variability between regions (F_{RT}=0.174), with only about 5% due to differentiation between populations within regions (Fig. 2c). This reflects the substantial difference between the Port Elizabeth (PE) population in the South Coast region and the three populations in the Western Overlap region. Although this species has a higher proportion of the total variance attributable to differences between populations than does *B. cincta* (18% as opposed to 12%), this amount was not found to be significant. This is due, in all likelihood, to the small number of populations sampled as well as the sample size of two of them.

The F_{PT} values varied considerably for the different loci, ranging from 0 to 0.503. A large portion of the overall differentiation between populations was due to two loci, *PGD* and *LAP*. For both, the differentiation within populations was almost equal to the differentiation between populations (F_{PT} = 0.494 and 0.503 respectively), both of which were significant ($p<0.001$), indicating that, at least at these two loci, the populations are clearly subdivided. Most of this was due to the differentiation between regions. Only two other loci had relatively high F_{PT} values, these

Table 11d. Hierarchical analysis of genetic differentiation between populations of *B. lagenaria* by Wright's *F*-statistics.

Locus	H_T	F_{PT}	F_{RT}	F_{PR}	1-F_{PT}
ARK	0.225	0.963 ***	0.963	0.012	0.037
AAT-2	0.061	0.165 *	-0.082	0.228	0.835
DIA-1	0.190	0.380 ***	-0.002	0.381	0.620
HEX-3	0.096	0.300 ***	-0.172	0.403	0.700
IDH-1	0.139	0.568 ***	0.568	0.000	0.432
IDH-2	0.118	0.110	-0.038	0.142	0.890
LAP	0.110	0.383 ***	-0.211	0.491	0.617
MPI	0.079	0.107	0.104	0.003	0.893
ODH	0.448	0.346 ***	-0.008	0.351	0.654
GL	0.217	0.143	-0.019	0.159	0.857
PHP	0.054	0.134	-0.091	0.206	0.866
PGD	0.306	0.153	0.112	0.046	0.847
PGM	0.538	0.514 ***	0.423	0.157	0.486
SDH	0.020	0.003	-0.003	0.000	0.997
over all loci		0.379 ***	0.194	0.230	0.621

For key to abbreviations see Table 11a.

being *GPI* and *ARK* (F_{PT} = 0.123 and 0.189 respectively), and again most of the variation was due to between-region differentiation.

Comparing *B. pubescens* with *B. cincta* and *B. papyracea*, the loci again show no patterns in levels of variability, or the source of this variability.

B. lagenaria

The *F*-statistics for the *B. lagenaria* populations presents a slightly different picture to the three species already discussed (Table 11d). There was polymorphism at fourteen loci, all of which were shared with at least one of the previous three species. The total expected heterozygosity for the loci ranged from 0.020 for *SDH*, to 0.538 for *PGM*. The proportion of the total genetic variance due to within-population differentiation, only about 62%, was relatively low compared to the above-mentioned species, as can be seen when Fig. 2d is compared with Figs 2a to 2c. The variation attributable to differentiation between populations, about 38%, is highly significant ($p<0.001$), indicating that populations of this species are strongly subdivided. Slightly more of this variation was due to variation between populations within regions (F_{PR} = 0.230), than between regions (F_{RT} = 0.194).

The contributions of the loci to the differentiation between populations, varied substantially, with more than half of the loci showing significant differentiation. There was almost no variation among populations at the *SDH* locus (F_{PT} = 0.003), whilst at the *ARK* locus almost all of the total variation at this locus was due to differentiation between populations (F_{PT} = 0.963). Less than 4% of the variation at this locus was due to differences within populations, and this can clearly be seen in Fig. 1d. ARK^{52} was the common allele in seven of the populations (it was fixed in six of these, and almost fixed in the other), but the eighth population, Durban (DN), was fixed for a different allele, ARK^{70}. As expected, all of this variation, was due to differentiation between regions, since the DN population was the only representative of the East Coast region. Other loci which

Table 11e. Hierarchical analysis of genetic differentiation between populations of *B.* sp. A by Wright's *F*-statistics.

Locus	H_T	F_{PT}	1-F_{PT}
ARK	0.022	0.000	1.000
AAT-2	0.490	0.136	0.864
HEX-3	0.022	0.000	1.000
IDH-1	0.302	0.000	1.000
MPI	0.085	0.036	0.964
GL	0.443	0.259 *	0.741
PHP	0.172	0.045	0.955
PGM	0.159	0.034	0.966
PGD	0.074	0.000	1.000
over all loci		0.112	0.888

For key to abbreviations see Table 11a.

Table 11f. Hierarchical analysis of genetic differentiation between populations of *B. catarrhacta* by Wright's *F*-statistics.

Locus	H_T	F_{PT}	1-F_{PT}
AAT-2	0.480	0.658 ***	0.342
GAP	0.492	0.136	0.864
HEX-3	0.498	0.051	0.949
PGM	0.500	0.312 *	0.688
over all loci		0.286	0.714

For key to abbreviations see Table 11a.

contributed to the high between-population differentiation, were *IDH-1*, *PGM*, *LAP* and *DIA-1* (F_{PT} = 0.568, 0.514, 0.383 and 0.380 respectively).

Once again, there was no correspondence between levels of heterozygosity, amount of between-population differentiation, and where this differentiation occurred.

***B.* sp. A**

Since there were only two *B.* sp. A populations sampled, the total genetic variation was broken down into only two components; within-population differentiation, and among-population differentiation (Table 11e). There were nine polymorphic loci, all of which were polymorphic in at least two of the other species discussed above. The heterozygosity levels of these loci ranged from 0.022 (*ARK*) to 0.490 (*AAT-2*).

The differentiation within populations accounted for about 89% of the total genetic variance, with about 11% (not significant) due to differentiation between the populations (Fig. 2e). Four of the loci had no, or negligible, variation between the populations. Of the other five loci, only two, *GL* and *AAT-2* (F_{PT} = 0.259 and 0.136 respectively), had relatively substantial amounts of variation between the populations, although only at the former was significant ($p < 0.05$) differentiation detectable. The amount of within-population differentiation for this species was similar to that for *B. cincta*, *B. papyracea*, and *B. pubescens*.

B. catarrhacta

As with the previous species, only two populations were sampled. The levels of total expected heterozygosity, and the amount of genetic differentiation within and between the populations are shown in Table 11f. Only four loci were polymorphic, one of which (*GAP*) was

Table 11g. Hierarchical analysis of genetic differentiation by Wright's F-statistics. The first column in each section shows the results for all of the populations in all of the species. The results in the second column are those calculated when the two *B. catarrhacta* populations were excluded. The values in bold indicate those loci which altered substantially when *B. catarrhacta* was excluded.

Locus	H_T		F_{PT}		F_{ST}		F_{PS}		1-F_{PT}	
ARK	0.627	0.582	0.571	0.509	0.318	0.300	0.371	0.298	0.429	0.491
AAT-1	0.116	0.006	**0.952**	**0.029**	**0.952**	**0.027**	0.002	0.002	0.048	0.971
AAT-2	0.269	0.241	0.434	0.372	0.244	0.254	0.251	0.159	0.566	0.628
DIA-1	0.462	0.436	0.732	0.699	0.685	0.647	0.149	0.145	0.268	0.301
G6PDH	0.111	-	1.000	-	1.000	-	0/0[1]	-	0.000	-
GPI	0.284	0.196	0.709	0.551	0.665	0.486	0.130	0.127	0.291	0.449
GAP	0.112	-	0.778	-	0.732	-	0.169	-	0.222	-
HEX-3	0.154	0.047	**0.589**	**0.198**	**0.510**	**-0.024**	0.161	0.217	0.411	0.802
IDH-1	0.175	0.185	0.513	0.509	0.338	0.338	0.264	0.259	0.487	0.491
IDH-2	0.038	0.040	0.108	0.107	0.006	0.008	0.103	0.100	0.892	0.893
LAP	0.743	0.714	0.802	0.781	0.636	0.602	0.456	0.450	0.198	0.219
MDH-1	0.290	0.203	0.808	0.708	0.805	0.703	0.017	0.016	0.192	0.292
MDH-2	0.305	0.219	1.000	1.000	1.000	1.000	0/0	0/0	0.000	0.000
MDH-3	0.112	0.001	**0.991**	**0.000**	**0.991**	**-0.002**	0.003	0.002	0.009	1.000
ME-3	0.111	-	1.000	-	1.000	-	0/0	-	0.000	-
MPI	0.719	0.687	0.656	0.617	0.634	0.593	0.061	0.059	0.344	0.383
ODH	0.580	0.529	0.472	0.385	0.358	0.266	0.177	0.162	0.528	0.615
GL	0.663	0.624	0.659	0.615	0.593	0.542	0.163	0.159	0.341	0.385
PHP	0.138	0.031	**0.812**	**0.110**	**0.791**	**0.017**	0.097	0.095	0.188	0.890
PGM	0.648	0.622	0.439	0.411	0.207	0.254	0.293	0.211	0.561	0.589
PGD	0.772	0.747	0.853	0.838	0.786	0.767	0.310	0.305	0.147	0.162
SDH	0.221	0.125	**0.529**	**0.112**	**0.494**	**0.049**	0.068	0.066	0.471	0.888
over all loci			0.683	0.599	0.567	0.496	0.267	0.206	0.317	0.401

H_T = total limiting variance (total gene diversity) across populations.

F_{PT} = proportion of total genetic variance attributable to population differentiation.

F_{ST} = proportion of total genetic variance attributable to differences between species.

F_{PS} = proportion of total genetic variance attributable to among-population within-species variation.

1-F_{PT} = proportion of total genetic variation attributable to differentiation within populations.

[1]0/0 = all populations are monomorphic, but not for the same allele.

- all populations are monomorphic for the same allele.

monomorphic in all of the other species. The total expected heterozygosity levels were high for all the loci (0.480 to 0.500). Over all four loci, the proportion of the total genetic variability attributable to within-population differentiation was approximately 71% (Fig. 2f), a level intermediate between that for *B. lagenaria* (62%) and the other species (82% to 93%). Although about 29% of the total variance was due to differentiation between populations, much higher than for the other species, excepting *B. lagenaria*, it was not found to be significant. Again, as with *B. pubescens*, the lack of significance was most likely due to the low number of populations/individuals sampled. However, *AAT-2* contributed significantly ($p<0.001$) to the amount of differentiation among populations, with more variation between the populations than within them (F_{PT} = 0.658). *PGM* also showed a significant ($p<0.05$) among-population differentiation (F_{PT} = 0.312).

All species

Wright's (1978) hierarchical *F*-statistics were calculated for all of the species combined (Table 11g, column 1). All of the populations belonging to the same species were grouped together. This analysis also included *B.* sp. B.

Twenty two of the 25 loci were polymorphic in at least one of the populations. The levels of the total expected heterozygosity ranged from 0.038 for *IDH-2*, to 0.772 for *PGD*. The proportion of the total genetic variation due to within-population differentiation was about 32% for all loci combined, with a corresponding 68% attributable to differentiation among populations. This latter value is much higher than the values obtained when the different species were analysed separately, and is highly significant ($p<0.001$). The much smaller proportion of within-population differentiation obtained when all populations and all species are compared, can be clearly seen by comparing Figs. 2a-f with Fig. 2g. The loci did not make equal contributions to the overall differentiation between the populations, but all were significant ($p<0.001$). The F_{PT} values ranged

from 0.108 for *IDH-2* and 0.434 for *AAT-2* (the two lowest values), through to 1.0 for three loci (*ME-3*, *G6PDH* and *MDH-2*). Only four loci contributed less than 50% to the among-population differentiation. An F_{PT} value of 1.0 indicates that the locus was monomorphic in all of the populations, but not all of the populations had the same allele. This results in zero differentiation within populations, and all of the differentiation residing between species.

As seen in Fig. 2g, a large portion of the genetic variation between populations was due to differentiation between the species (F_{ST} = 0.567), and less due to differentiation among populations within species (F_{PS} = 0.267).

Since *B. catarrhacta* appears to be well differentiated from the other species, a separate analysis was performed which excluded this species. The results are also given in Table 11g (column 2 for each parameter). Although the overall F_{PT} was somewhat reduced (compare Fig. 2g with Fig. 2h), the value of 0.599 still indicates a large, significant ($p<0.001$) amount of differentiation between the populations of the other species. Also, as before, a large proportion of the differentiation was due to differences between species (F_{ST} = 0.496), with less due to differences between populations within species (F_{PS} = 0.206). At a few of the loci, however, the effect of excluding the two *B. catarrhacta* populations was dramatic (these results are highlighted in Table 11g). For example, in the case of *MDH-3*, the F_{PT} value dropped from 0.991 to 0.000, indicating that the *B. catarrhacta* populations were responsible for all of the differentiation between the species at this locus. For similar reasons, four other loci (*AAT-1*, *HEX-3*, *PHP* and *SDH*) also showed big drops in the F_{PT} values when *B. catarrhacta* was excluded. A further indication of the difference between *B. catarrhacta* and the other species was that the number of polymorphic loci decreased from 22 to 19 with the exclusion of *B. catarrhacta*.

The results of the above analyses imply that *B. catarrhacta* is well differentiated at some loci, whereas the rest of species are not at all differentiated at these loci. However, there are many other loci that do differentiate between the other species.

Genetic identity and distance

Unlike Wright's *F*-statistics dealt with above, several other statistics can include the monomorphic loci in estimations of genetic differentiation between pairwise comparisons of taxa, and thus give a better measure of overall genetic differentiation. These include Nei's (1978) unbiased coefficients of distance (*D*) and identity (*I*), and Rogers' modified distance (*D* - Wright, 1978), which were calculated for all pairwise comparisons of the 34 populations (Appendices C and D). Nei's *D* ranges from zero when there is no genetic difference between two taxa, to infinity, when the two taxa are completely different, whilst Rogers' *D* ranges from zero to one. Nei's *I* ranges from one when there is 100% genetic similarity between the taxa, to zero when there is no similarity. Much of the literature documenting these types of data uses Nei's *D*, and for ease of comparisons, this is the statistic used below, unless otherwise indicated.

In a survey of the literature Thorpe (1983) found that in comparisons between over 7000 conspecific populations (of plants and animals), about 98% of Nei's *D* estimates were less than 0.10, whereas between 900 congeneric comparisons, most *D* estimates were between 0.16 and 1.0. Thorpe found that there did not appear to be much difference in these estimates between major taxa, including invertebrates, except for birds. Woodruff *et al.*, (1988) note that, between sexually reproducing molluscs, the majority of *D* estimates between conspecifics are less than 0.10, whilst most congeneric distances were more than 0.05, and usually between 0.20 and 0.60.

The populations sampled were grouped together in a nested hierarchy, and the *D* estimates were averaged by region within each species. These are given in Table 12. Setting aside the three species which had only one or two populations, the populations within regions were always more similar to one another than to populations within other regions, averaging 0.000 for three West Coast *B. papyracea* populations and three Western Overlap *B. pubescens* populations. Furthermore, with a single exception, similarity decreased as distance between regions increased.

Table 12. Mean and range of Nei's D averaged by region within each of the species.

Species	Region	N[1]	Region: West Coast	Western Overlap	South Coast	East Coast
B. cincta	WC	4	0.008 0.002-0.013			
	WO	4	0.018 0.011-0.027	0.005 0.002-0.009		
	SC	2	0.027 0.015-0.048	0.047 0.041-0.058	0.021 0.021-0.021	
B. papyracea	WC	3	0.000 0.000-0.001			
	WO	4	0.011 0.003-0.022	0.012 0.000-0.024		
B. pubescens	WO	3		0.000 0.000-0.000		
	SC	1		0.089 0.075-0.103	nc[2]	
B. lagenaria	WC	3	0.024 0.021-0.026			
	WO	3	0.028 0.011-0.045	0.017 0.013-0.026		
	SC	1	0.049 0.044-0.052	0.035 0.019-0.063	nc	
	EC	1	0.127 0.123-0.134	0.106 0.086-0.140	0.080 0.080-0.080	nc
B. sp. A	WC	2	0.018 0.018-0.018			
B. catarrhacta	WC	1	nc			
	WO	1	0.050 0.050-0.050	nc		

N[1] = number of populations.
nc[2] = no comparisons.

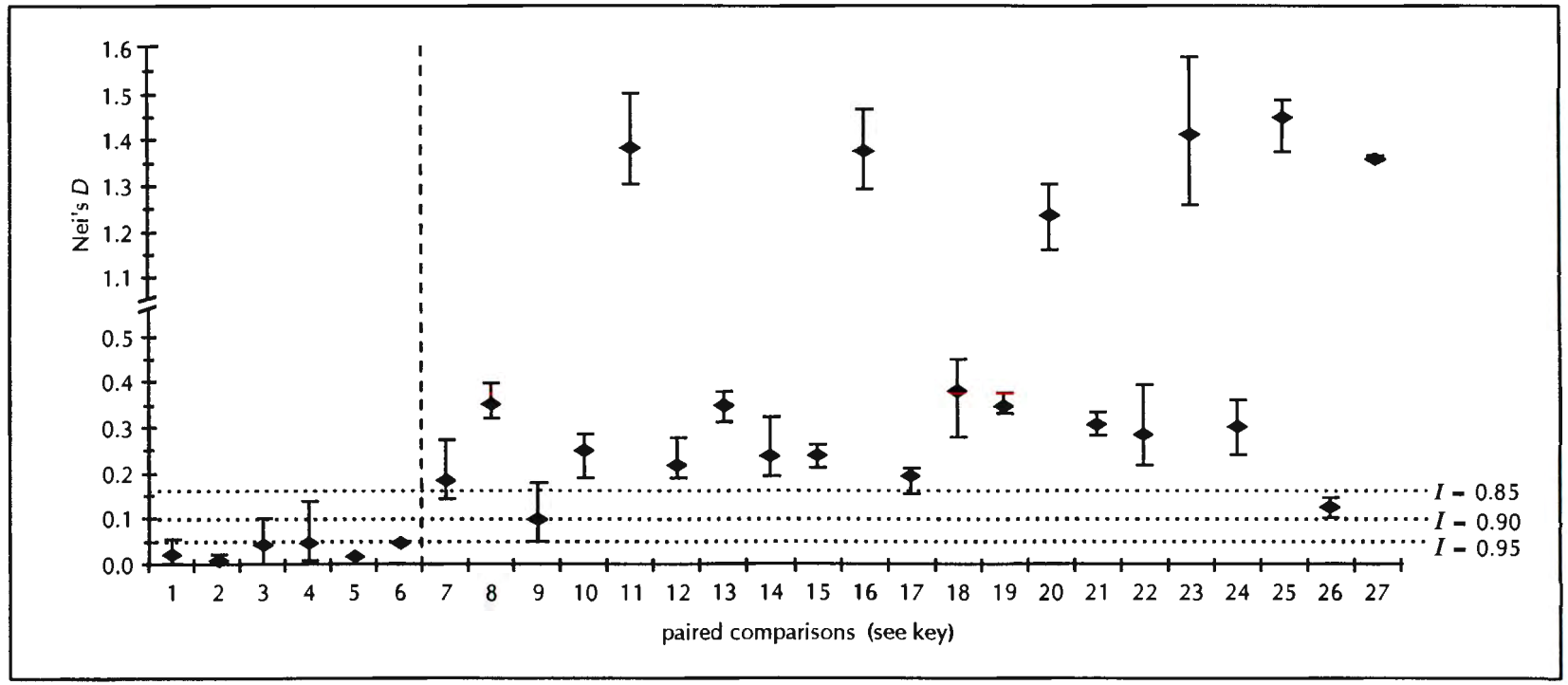
The levels of intra- and inter- specific differentiation can be examined when the distance coefficient is averaged by species (Table 13 and Fig. 3). Within species, Nei's *D* values ranged from zero to 0.140, with the means covering between 0.010 for the *B. papyracea* populations and 0.050 for the *B. lagenaria* and *B. catarrhacta* populations. The large range in *D* within the *B. pubescens* populations (Fig. 3) was due to the relatively high *D* values between the Port Elizabeth population (South Coast) and the other populations (Table 12), which are geographically separated by at least 600km. Likewise, for *B. lagenaria*, the large range in *D* between populations was due to the Durban (East Coast) population, which is separated by at least 1280km from the other populations. Excluding these two populations, the largest *D* between any two populations belonging to the same species, was reduced to 0.063 (i.e. between the Sparks Bay and Mossel Bay populations of *B. lagenaria* - Table 12).

Between species, Nei's *D* values ranged from 0.054 (between *B. cincta* and *B. lagenaria*; Table 13, Fig. 3) to 1.583 (between *B. catarrhacta* and *B. lagenaria*), with the means ranging from 0.100 (between *B. cincta* and *B. lagenaria*) to 1.449 (between *B. catarrhacta* and *B.* sp. A). It is significant to note that the *D* values between the two *B. catarrhacta* populations and all of the other populations, were greater than 1.15 (Table 13, Fig. 3). Excluding the *B. catarrhacta* populations, the highest *D* value was 0.450 (between *B. lagenaria* and *B. pubescens*). It is clear that the two *B. catarrhacta* populations are well differentiated from the other populations, due mainly to 13 loci which were fixed for unique alleles in this species. The *D* value of 1.15 separating *B. catarrhacta* is well above the value normally associated with congeneric comparisons and implies that this species could be placed in a separate genus. The lowest interspecific genetic distances were between the *B. cincta* and *B. lagenaria* populations (No. 9 in Fig. 3). There was even an overlap between the range of *D* values derived from comparisons of these two species, and the ranges recorded between populations within each of these species (Nos. 1 and 4 in Fig. 3), indicating the overall similarity of these two species relative to the other species. The genetic distances between *B.* sp. A and *B.* sp. B (No. 26 in Fig. 3) were also relatively low, being more similar to those between *B. cincta* and *B. lagenaria* than between the other species.

Table 13. Mean and range of Nei's *D* averaged by species.

Species	N[1]	CIN	PAP	PUB	LAG	A	CAT	B
B. cincta	10	0.022 0.002-0.058						
B. papyracea	7	0.188 0.148-0.272	0.010 0.000-0.024					
B. pubescens	4	0.356 0.321-0.397	0.350 0.315-0.380	0.045 0.000-0.103				
B. lagenaria	8	0.100 0.054-0.175	0.241 0.195-0.324	0.378 0.282-0.450	0.050 0.011-0.140			
B. sp. A	2	0.250 0.192-0.284	0.241 0.212-0.263	0.349 0.334-0.375	0.284 0.222-0.392	0.018 0.018-0.018		
B. catarrhacta	2	1.386 1.312-1.502	1.378 1.301-1.466	1.240 1.168-1.305	1.413 1.268-1.583	1.449 1.383-1.488	0.050 0.050-0.050	
B. sp. B	1	0.222 0.191-0.278	0.194 0.159-0.210	0.309 0.286-0.333	0.303 0.246-0.358	0.126 0.105-0.147	1.359 1.354-1.365	nc[2]

N[1] = number of populations.
nc[2] = no comparisons.



Key:

Within species:		Between species:					
1	CIN - CIN (10)	7	CIN - PAP	14	PAP - LAG	21	PUB - B
2	PAP - PAP (7)	8	CIN - PUB	15	PAP - A	22	LAG - A
3	PUB - PUB (4)	9	CIN - LAG	16	PAP - CAT	23	LAG - CAT
4	LAG - LAG (8)	10	CIN - A	17	PAP - B	24	LAG - B
5	A - A (2)	11	CIN - CAT	18	PUB - LAG	25	A - CAT
6	CAT - CAT (2)	12	CIN - B	19	PUB - A	26	A - B
		13	PAP - PUB	20	PUB - CAT	27	CAT - B

Figure 3. Mean and range of Nei's genetic distance within and between species. Note break in the Y axis. The three horizontal lines show Nei's identity (*I*) values. Error bars represent the range. Figures in parentheses indicate numbers of populations of each species.

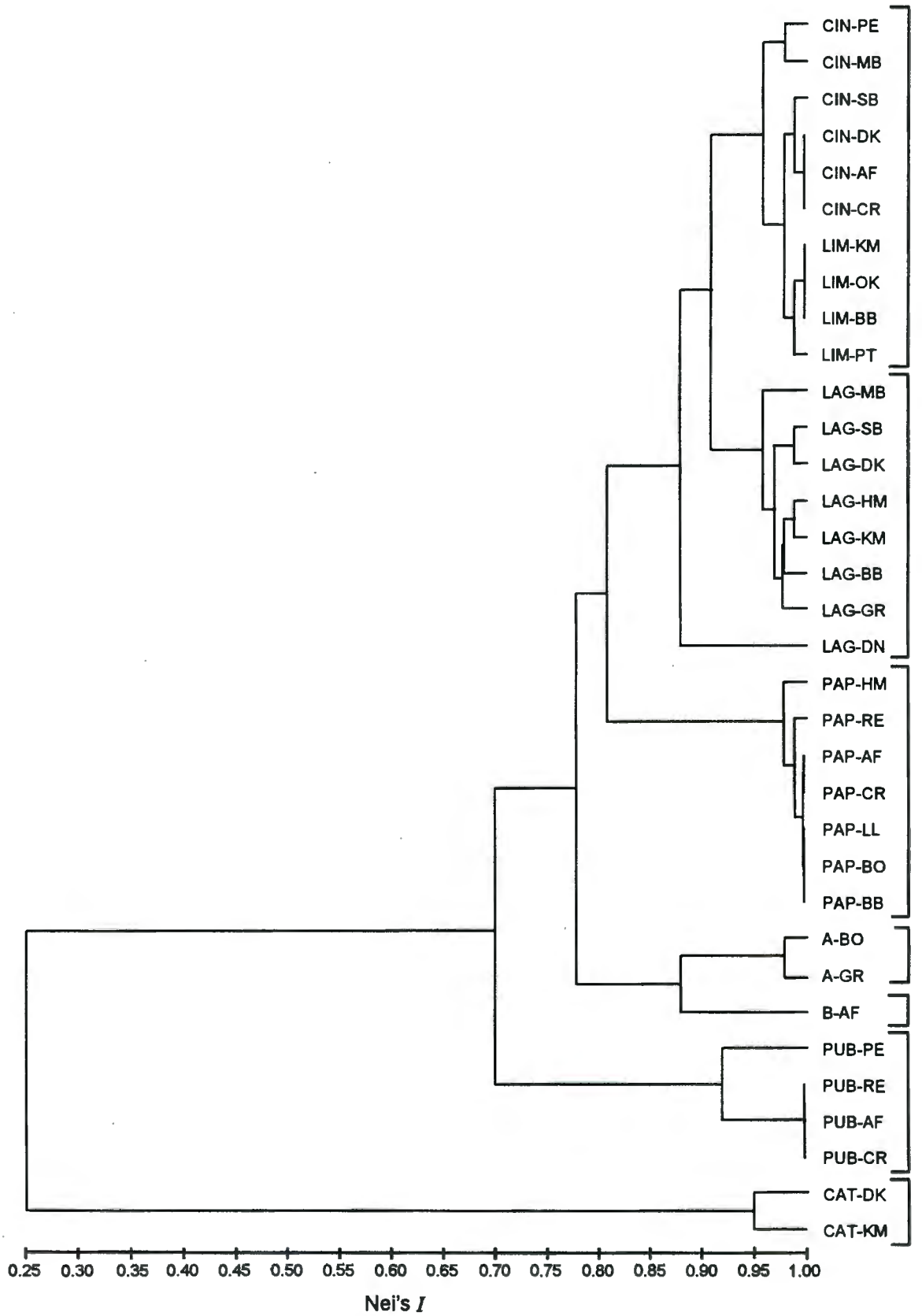


Figure 4. Phenogram showing similarities of the 34 *Burnupena* populations using Nei's *I* and UPGMA clustering. See Table 1 for population abbreviations.

A phenogram based on Nei's *I*, and constructed by the unweighted pair group method of analysis (UPGMA) indicates the similarity of the populations to each other (Fig. 4). The topology of the tree produced using Nei's *D*, was identical to that generated using Nei's *I*. The goodness of fit statistics were, however, better with the latter, where Prager and Wilson's (1976) "*F*-value" was 2.365, and the cophenetic correlation was 0.993. These statistics give an indication as to how well the output matrix, which is produced by adding together the distances between each of the populations, fits the input matrix which is used to construct the phenogram. The lower the *F*-value, the better the fit of the tree to the data.

The most striking feature to emerge is that the two *B. catarrhacta* populations were clearly well separated from the remaining populations (Fig. 4).

With the exception of the Durban *B. lagenaria* population (LAG-DN), it is also evident that all of the populations belonging to the same species clustered together, before being joined to other species. The LAG-DN population was only joined to the other *B. lagenaria* populations after these had been joined to the cluster of *B. cincta* populations. It is of particular interest that all of the remaining populations of *B. lagenaria* and *B. cincta* are separable at the specific level despite substantial geographic overlap, since they are among the species pairs that have been confused on morphological grounds.

In the case of species which were sampled at a large number of sites (*B. cincta*, *B. lagenaria* and *B. papyracea*), it is apparent from the phenogram that the populations within a given region tended to cluster together. The PUB-PE population was quite distinct from the others in its species, only joining the remaining *B. pubescens* populations at a clustering level (I = 0.915) just less than that uniting the *B. lagenaria* (excluding LAG-DN) and *B. cincta* populations (I = 0.909).

An arbitrary cut-off line at an I value of 0.85 (D=0.163), would recognise five species, or "higher" taxa. The *B. cincta* populations, and all of the *B. lagenaria* populations would be grouped together as one species. Likewise, the two *B.* sp. A populations would be grouped together with *B.*

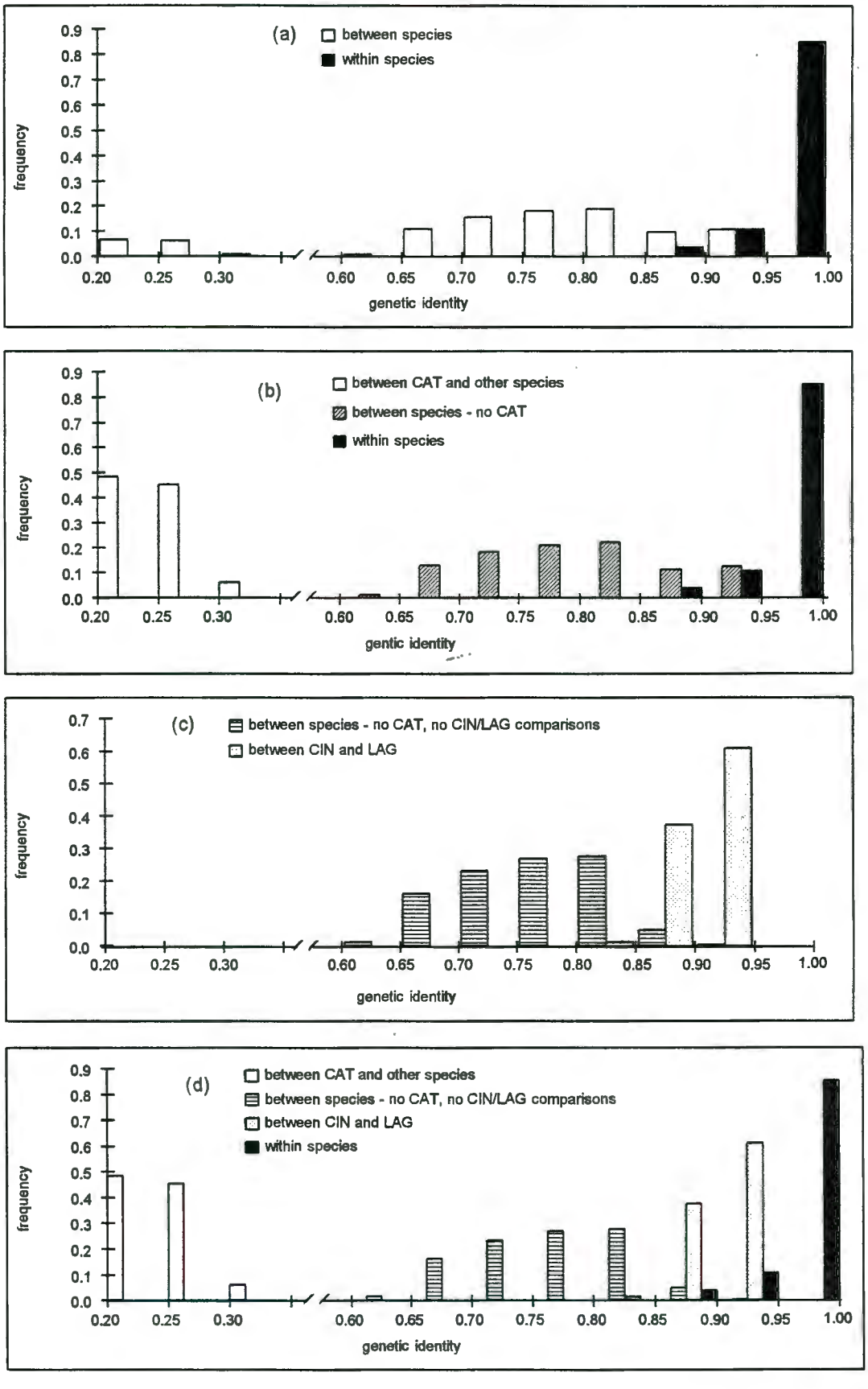


Figure 5. Frequency distributions of Nei's genetic identity (I) between different sets of populations of *Burnupena*. See Table 1 for species abbreviations.

sp. B. The other three species would be *B. papyracea*, *B. pubescens* and *B. catarrhacta*. In an earlier survey, Thorpe (1982) found that for conspecific populations, 98% of Nei's *I* values exceeded 0.85, that 93% of the values exceeded 0.90, and that about 80% were above 0.95. Taking a cut-off level at $I = 0.90$ ($D=0.105$), would recognise seven entities. The *B. cincta* and *B. lagenaria* populations would still be grouped together. The LAG-DN population would constitute a separate taxon. The *B.* sp. A populations and *B.* sp. B would be split into two species, and the other three species would remain distinct. A cut-off level at $I = 0.95$ ($D=0.051$), would separate *B. cincta* and *B. lagenaria*, the PE population of *B. pubescens* also being split off from the other *B. pubescens* populations.

A series of frequency distributions of genetic identity (Nei's *I*) comparisons between different sets of populations are shown in Fig. 5. Such analyses have been done by a number of authors (Avise, 1975; Thorpe, 1982, 1983; Johnson et *al.*, 1986) to illustrate the ranges of *I* between populations at different taxonomic levels. The identities were grouped into increments of 0.05, and the distribution shows the frequency of *I* values in each increment. Fig. 5a shows two distributions, that between all populations of different species (congeneric species), and that between all populations within the same species (conspecifics). Over 85% of the within-species *I* values are above 0.95, with more than 95% above 0.90, and none below 0.85. These percentages are slightly higher than those of Thorpe (1982, see above) and Johnson et *al.* (1986) for land snails of the genus *Partula*. There is an overlap between the two distributions between the 0.85 and 0.94 levels. All of the conspecific comparisons that fall in the 0.85 to 0.89 increment are between the LAG-DN population and other *B. lagenaria* populations.

Another point of interest is the gap in *I* values between 0.35 and 0.60 - none of the comparisons between species yielded *I* values in this range. The data can be broken down into (a) comparisons between *B. catarrhacta* populations and other populations, and (b) comparisons between the other populations excluding *B. catarrhacta*. These data are shown in Fig. 5b, and they show clearly that the left hand tail in the distribution in Fig. 5a is due to comparisons between *B. catarrhacta* and other populations. This again emphasises the distinctly different nature of *B. catarrhacta*.

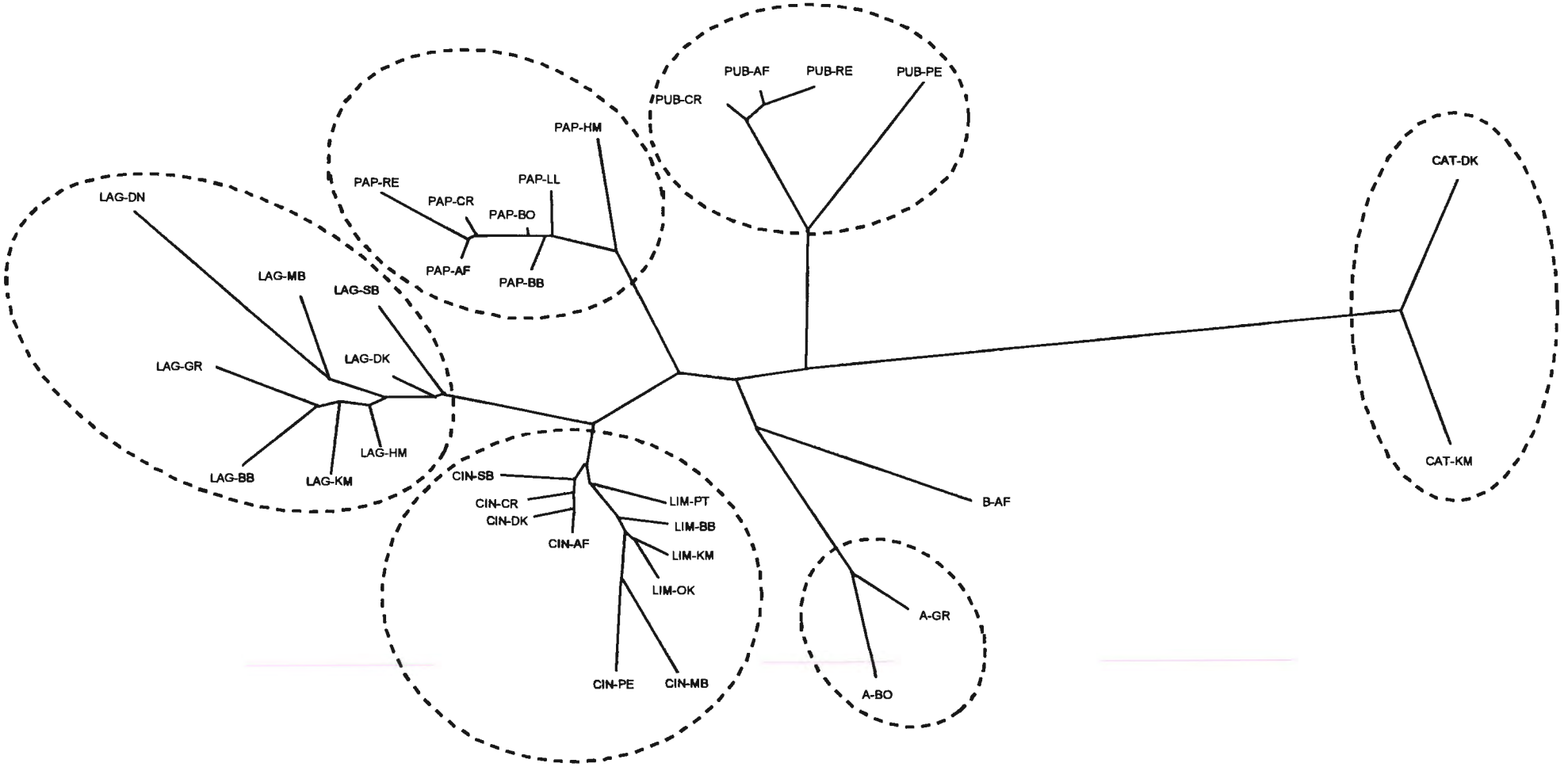


Figure 6. Unrooted Wagner network of Rogers' distance illustrating the relationships between the 34 populations of *Burnupena*. See Table 1 for population abbreviations.

At the other end of the scale, the region of overlap between conspecifics and congenerics can be examined more closely. *B. cincta* and *B. lagenaria* species are very similar relative to the other species. Fig. 5c shows the distributions obtained when comparisons are made (a) between populations of these two species only, and (b) between populations in all other species excluding *B. cincta/B. lagenaria* comparisons (but including comparisons of *B. cincta* and *B. lagenaria* with other species). Comparisons with *B. catarrhacta* have been omitted. It is evident that the *B. cincta/B. lagenaria* comparisons are responsible for almost all of the right hand tail of the between-species comparisons in Fig. 5b.

The various distributions described above are shown together in Fig. 5d, emphasising that most of the overlap between conspecific and congeneric comparisons is due to the comparisons between the populations of *B. cincta* and *B. lagenaria*.

Rogers' modified distance (Wright, 1978) was calculated for all pairwise comparisons, and the distance matrix was converted into an unrooted Wagner network using the distance Wagner procedure of Farris (1972). This method does not assume a constant evolutionary rate across all lineages, an assumption required for the UPGMA.

Swofford's (1981) "multiple addition criterion" (MAC) was used as the criterion for the selection of the next OTU to be added to the network. During each cycle, a number of partial networks are generated, and the goodness-of-fit criterion used for selecting those partial networks to be saved for the next cycle, was the "*F*-value" of Prager and Wilson (1976). Two networks were produced, which differed only in that they did not join LIM-KM with LIM-OK. These were linked, one at a time, to the CIN-PE-CIN-MB cluster. In all other respects, the two networks were identical.

Of the two networks produced, the one presented (Fig. 6) had slightly better goodness-of-fit statistics ($F = 3.691$, cophenetic correlation $= 0.994$). The phenogram produced using Nei's I and

UPGMA had a better *F*-value (2.396), than either of the networks produced by the distance Wagner procedure. This does not imply, however, that the former procedure is superior.

Examination of the Wagner network (Fig. 6) shows that all of the populations of the same species cluster together. This includes the LAG-DN population, which was not clustered together with the other *B. lagenaria* populations in the UPGMA phenogram. This difference aside, and excluding the details of the internal linkages within the species clusters, there was no difference between the Wagner network and the UPGMA phenogram.

A comparison of the internal linkages within the species clusters between the UPGMA and Wagner trees, reveals both differences and similarities. In both trees, the *B. catarrhacta* populations are well separated from the rest of the species, as would be expected from the initial allele frequencies. The clustering of the *B. pubescens* populations, and the placement of *B.* sp. B next to *B.* sp. A, are also identical. *B. cincta* and *B. lagenaria* are similarly associated more closely with each other rather than with any other species. The differences lie within the details of the *B. cincta*, *B. papyracea* and *B. lagenaria* species clusters. The major difference between the two trees was the placement of the LAG-DN population. As mentioned above, this population was placed within the *B. lagenaria* cluster in the Wagner network, but outside the cluster in the UPGMA tree.

The clustering with Nei's *I* using the UPGMA, and Rogers' *D* with the Wagner procedure, was repeated using matrices where all of the populations in the same species were pooled. Both methods produced identical topologies, with the positions of the species relative to one another remaining the same as that shown in Figs 4 and 6 (i.e. UPGMA and Wagner tree of all populations).

Sympatric populations

In the previous sections, the amount of genetic variation between all of the populations, averaged over all loci, showed that most of the species are fairly well differentiated from one

another. However, the existence of a certain level of genetic differentiation between any two populations does not in itself imply that they are reproductively isolated.

At nine of the localities, populations of two or more species were collected, and hence it was possible to look for evidence of gene flow between these sympatric populations. Since *B. catarrhacta* is clearly well differentiated from the other species, it has been excluded from this analysis, although it was sympatric with both *B. cincta* and *B. lagenaria* at both localities from which it was collected. A total of 18 separate comparisons were made with ten different species combinations (Table 14). In all of the comparisons, with the single exception of the *B. papyracea* and *B. lagenaria* populations at Blouberg (BB), the species partially or completely overlapped one another and were therefore clearly sympatric. Three criteria were used to indicate an absence of gene flow between the populations. Firstly, loci at which the two populations had no alleles in common. Secondly, loci where an allele was found in both of the populations, but occurred at a frequency of less than 0.01 in one of the populations and was relatively common in the other population. The third criterion used genotypes, rather than alleles, to determine possible lack of gene flow. By this criterion, alleles may be shared at the locus, usually at a low frequency in one population (but reaching 0.258 in one case), but there will be no shared genotypes. Those loci showing no gene flow by the first criterion, would also obviously fit the third criterion, since an absence of shared alleles would result in zero genotypic overlap.

At all of the sites where comparisons were made, there was at least one locus at which there appeared to be no gene flow between the pairs of species. There was only one comparison for which no locus was found using the first criterion, which is obviously the most stringent in terms of the probability that there is no gene flow. This was the comparison between the *B. cincta* and *B. lagenaria* populations at Dalebrook (DK). However, it is likely that there is no gene flow between these two populations at the *LAP* locus. At this locus, they share the LAP^{109} allele, but *B. lagenaria* was fixed for this allele, and the frequency in the *B. cincta* population was only 0.007, with one heterozygous individual having this allele. Consequently, there was no observed overlap of genotypes at this locus, since the only genotype in the *B. lagenaria* population was LAP^{109}/LAP^{109},

Table 14. Loci showing the absence of gene flow between sympatric populations, as indicated by: (a) lack of shared alleles, (b) situations where the frequency of a shared allele is less than 0.01 in one of the populations, and (c) absence of overlap of genotypes.

Sympatric species	site	no alleles shared	alleles < 0.01	genotypic overlap absent
B. cincta	BB	*PGD*	-	-
&	KM	*PGD*	-	-
B. lagenaria	DK	-	*LAP*	*LAP*
	MB	*LAP*	-	-
B. cincta	BB	*PGD, MPI*	-	-
&	CR	*PGD, MPI*	-	*ARK, GL*
B. papyracea	AF	*PGD, MPI*	-	*ARK*
B. cincta	CR	*DIA-1, MDH-2*	-	*MDH-1*
&		*PGD, PGM, GL,*		
B. pubescens	AF	*DIA-1, MDH-2*	-	*PGD, PGM*
		GL		
B. cincta &	AF	*LAP*	-	*GOT-2, DIA-1, GL*
B. sp. B				
B. papyracea &	BB	*PGD, MPI*	-	*LAP, ARK, GL*
B. lagenaria				
B. papyracea	CR	*PGD, MPI, MDH-2*	-	*ARK*
&	AF	*PGD, MPI, MDH-2*	-	*ARK*
B. pubescens	RE	*PGD, MPI, MDH-2*	-	*MDH-1, GPI, PGM*
		LAP, ARK		
B. papyracea &	BO	*MPI, LAP*	-	*PGD*
B. sp. A				
B. papyracea &	AF	*PGD, LAP*	-	*ARK*
B. sp. B				
B. pubescens &	AF	*GOT-2, MPI,*	-	*PGM*
B. sp. B		*LAP, MDH-2*		
B. lagenaria &	GR	*PGD, MPI,*	-	-
B. sp. A		*LAP*		

whilst the *B. cincta* population had four genotypes, namely, LAP^{100}/LAP^{100}, LAP^{100}/LAP^{106}, LAP^{100}/LAP^{109}, and LAP^{106}/LAP^{106}. The observation that this *B. cincta* population was not in Hardy-Weinberg equilibrium at this locus (Table 3), does not alter this conclusion, particularly because the lack of equilibrium was due to a deficit of the LAP^{100}/LAP^{106} genotype. In the three other comparisons between *B. cincta* and *B. lagenaria* populations, there was also only one locus at which no gene flow was apparent (Table 14).

For all comparisons between other species there were between three and eight loci showing an absence of gene flow between the sympatric populations.

DISCUSSION

Intraspecific genetic variation

Although the mean number of alleles per locus gives some idea of the amount of genetic variation present in a sample, it is not a very useful measure since it is highly dependant on the size of the sample. There are many low-frequency alleles in natural populations (Nei, 1987), and therefore the larger the sample size, the greater the chance of detecting more alleles. This trend can be seen in Table 4, if the mean sample size per locus is compared with the mean number of alleles per locus. Thus, as a measure of genetic variability, it is only meaningful when comparing samples of similar sizes. However, a number of observations can be made using these values. The four populations of *B. pubescens* appear to have had the greatest genetic variation (mean of 1.6). Even the two populations that had fewer than 10 individuals (PUB-RE and PUB-PE) had relatively high numbers of alleles per locus (1.4 and 1.5), even when compared with some of the other populations of larger sample size. The B-AF population also appears to have had a relatively high variation using this measure. It had a value of 1.5, almost the same as that averaged over the *B. cincta* populations (1.51), although it has a smaller sample size (18.7 versus 33.0 respectively). *B.* sp. A, *B. lagenaria* and *B. catarrhacta* tended to have the smallest amount of variation (1.35, 1.26 and 1.15 respectively), whilst *B. cincta* and *B. papyracea* were intermediate (1.51 and 1.41 respectively).

A commonly used measure of the amount of genetic variation present in a population is the proportion of loci that are polymorphic. However, care must be exercised when making comparisons with other studies reported in the literature, due to differences in the criterion used to define a polymorphic locus, and the number of loci examined.

The percentage of polymorphic loci within populations of *Burnupena* species ranged from 12 to 56%, with the means for each species ranging from 14 to 44% (Table 4). The levels of polymorphism within each of the species were, however, quite variable. For example, the

populations of *B. papyracea* ranged in polymorphism from 20 to 44%. Likewise, *B. cincta* populations ranged from 20 to 40%, *B. pubescens* populations from 32 to 56%, and *B. lagenaria* from 16 to 32%. As with the number of alleles per locus, the *B. pubescens* populations and the new species (both averaging 44%) exhibited the greatest polymorphism, whilst *B. catarrhacta* (14%) and some of the *B. lagenaria* populations (mean of 23.5%) appear to have had the least genetic variability. These values are only slightly affected (changing by < 5%) by the exclusion of populations in which only a few individuals were examined. The only exception was *B. pubescens* for which the mean value increased from 44 to 52% after exclusion of small samples. However, the overall patterns of genetic variability are not changed even after these adjustments.

In a review of enzyme variation in marine molluscs, Berger (1983) used the criterion that a locus was considered polymorphic if the most common allele occurred at a frequency of less than 0.98. In studies where at least 20 loci were examined, Berger found the proportions of polymorphic loci ranged from 20 to 60%. In two studies of whelks in the genus *Nucella*, polymorphism ranged from 10.5 to 21.1% for *N. emarginata* (Palmer, *et al.*, 1990), and from 44 to 67% in *N. lapillus* (Day & Bayne, 1988). In both cases 18 loci were examined, and the criterion used for polymorphism was the presence of more than one allele (the results from Day & Bayne were recalculated from their data since they presented their results using the 0.95 criterion). Thus the extent of polymorphism in *Burnupena* is within the normal range for marine molluscs in general, and for whelks in particular.

A disadvantage of using the proportion of polymorphic loci to estimate genetic variability is that this measure does not take into account the frequency of the alleles. For example, consider two populations in each of which a particular locus is polymorphic having two alleles. In the one population the frequencies might be 0.95 and 0.05, whilst in the other population they may be 0.55 and 0.45. It is obvious that the second population is more variable than the first, but just using the proportion of polymorphic loci will not reveal this. Because of this problem, a more informative measure of the extent of genetic variability is the frequency of heterozygotes, usually expressed as the average heterozygosity per locus.

The heterozygosity levels in this study ranged from 0.044 to 0.152, with the means per species ranging from 0.052 to 0.117 (Table 4). As with the proportion of polymorphic loci, the level of heterozygosity varied between populations within a species. For example, values ranged from 0.044 to 0.132 for *B. cincta*, and from 0.069 to 0.152 for *B. pubescens*. These ranges are however reduced, if the populations with smaller sample sizes are excluded (becoming 0.094 to 0.132 in *B. cincta* and 0.125 to 0.152 in *B. pubescens*).

Heterozygosity levels vary a great deal in marine molluscs. In a review of studies of the genus *Littorina*, Ward (1990) found that mean heterozygosity levels ranged from 0.022 to 0.381 if 15 or more loci were screened. Studies of *Haliotis rubra* (Brown, 1991) and *Crepidula fornicata* (Hoagland, 1985), document heterozygosity levels ranging from 0.099 to 0.150 and 0.034 to 0.062 respectively. Day and Bayne (1988) found in a survey of four populations of the whelk *Nucella lapillus*, that heterozygosity levels (based on 18 loci) ranged from 0.043 to 0.104. In a subsequent study, 21 populations of *N. lapillus* (of the same karyotype) had heterozygosities (based on 16 loci) ranging from 0.044 to 0.171 (Day *et al.*, 1993). Also using 18 loci , Palmer *et al.* (1990) observed heterozygosity levels ranging from 0.009 to 0.041 for six populations of *N. emarginata*. As with polymorphism, heterozygosity levels in *Burnupena* are thus very similar to the levels observed in other whelks.

All three measures of genetic variation show the same general patterns. They indicate that *B. pubescens* is the most variable species, having the highest value for mean number of alleles per locus (1.6), percentage polymorphism (44) and average heterozygosity (0.117). *B. catarrhacta*, *B. lagenaria* and *B.* sp. A exhibit the least amount of variation, and *B. cincta*, *B. papyracea* and the new species have intermediate levels of variability (Table 4).

It has been shown that heterozygosity levels are related to population size (Soule, 1976; Nei, 1987), with small, isolated, populations tending to have lower heterozygosity levels. Populations that have recently diverged, or gone through a "bottleneck" may also exhibit low

heterozygosity values (Soule, 1976). There have also been many studies, mostly on bivalves, that have tried to relate heterozygosity levels to components of fitness such as survival and growth rate (Zouros et *al.*, 1980; Gaffney & Scott, 1984; Koehn & Gaffney, 1984; Allendorf & Leary, 1986; Gosling, 1989; Skibinski & Roderick, 1989; Volckaert & Zouros, 1989; Bricelj & Krause, 1992). The results have been mixed, with some studies reporting a positive correlation between multiple-locus heterozygosity and growth rate, whilst others found no significant correlation.

B. catarrhacta, *B. lagenaria* and *B.* sp. A all have relatively low heterozygosity values (0.057, 0.057 and 0.052 respectively). Since the two populations of *B. catarrhacta* have large genetic distance values when compared with all of the other populations, indicating that this species has been separated from the others for a long time, their low heterozygosity levels are most likely due either to small population size, or to a recent bottleneck event. Although population sizes have not been quantified, this species does appear to have relatively smaller population sizes when compared with the other species (personal observation). *B. lagenaria* and *B.* sp. A on the other hand, show much lower genetic distance values with the other species, relative to those exhibited between *B. catarrhacta* and other species. This, together with lower heterozygosity values, might imply a more recent divergence, although population size and recent bottlenecks cannot be excluded as possible factors determining the heterozygosity level. The population sizes of *B. lagenaria* do, however, appear to be large, since this species is generally very abundant wherever it occurs, and it also has a wide distribution. This implies that recent divergence is a more likely explanation for the lower heterozygosity in this species, especially when its low genetic distances from *B. cincta* are considered. Population sizes of *B.* sp. A on the other hand appear to be smaller (personal observation), increasing the likelihood that this is responsible for the low heterozygosity in this species.

Although the heterozygosity levels for the other species, namely *B. papyracea*, *B. cincta*, *B. pubescens* and the new species, are higher than those for the three species discussed above, none of them has high values relative to reported values for marine molluscs. Population sizes for the first

three species appear to be fairly large, whilst the new species is scarce and probably has small population sizes.

When the heterozygosity levels were considered locus by locus in each of the populations of each species, the loci were very variable. This reinforces the statement that it is important to examine a large number of loci, since the interlocus variance is very large.

Although the species differed in their amounts of variation, the results of the *F*-statistics indicated that in all species, the proportion of the total genetic variation attributable to differentiation between individuals within populations, was greater than that due to differentiation between populations. For most species, variation within populations, averaged over all loci, accounted for over 80% of the total variation, only *B. lagenaria* and *B. catarrhacta* having slightly smaller proportions (62% and 71% respectively). Although the loci varied greatly in their amounts of variation, in only a few cases was there more differentiation between populations than within populations (two loci in *B. pubescens*, three in *B. lagenaria* and one in *B. catarrhacta*).

The amount of genetic variation within populations ranged widely, with some populations having very little variability, others being highly variable. Also, the amount of genetic variation found in populations within a given species was not uniform over its range. When the levels of genetic variation were averaged over the populations within each species, the different species of *Burnupena* still exhibited different amounts of genetic variation, although the reasons for this remain speculative. Nevertheless, the extent of the genetic variation within this genus appears to be within the normal range reported for marine molluscs in general, and for whelks in particular (Berger, 1983; Day & Bayne, 1988; Ward, 1990; Palmer *et al.*, 1990; Day *et al.*, 1993).

Population structure and intraspecific genetic differentiation

The amount of differentiation between populations within a species needs to be considered to put into perspective the differences between species. As mentioned above, the amount of genetic variation observed in the populations within a species is not uniform across the ranges sampled. There are also differences in alleles and allele frequencies between the populations. These differences can be observed when geographic variation in allele frequencies, and the results of Wright's hierarchical *F*-statistics, are examined. These two analyses consider only polymorphic loci. Measures of genetic distance quantify the degree of differentiation between populations, and can also be used to indicate which populations are least or most differentiated from other populations. Nei's genetic distance, *D*, is computed using all of the loci examined, not only the polymorphic ones, thereby giving a better measure of overall genetic differentiation.

It is evident from Table 12 that there are significant differences between some or all of the populations within the species, for at least some of the loci. All of the six species that were tested for differences in allele frequency of polymorphic loci showed significant differences between populations when summed over all loci (Table 10a). Using the *F*-statistics, again, all six species had at least one polymorphic locus that showed a significant difference between populations. However, when taken over all loci, only two species, *B. cincta* and *B. lagenaria*, showed significant differences between the populations (Table 11a,d). Although there is a difference in the sensitivity of the two methods, it is clear that all of these six species do show at least some differentiation between populations. In the following discussion, each species will be considered separately, followed by comments on the genus as a whole.

B. cincta

The results of the contingency table analysis show not only a significant overall heterogeneity between all populations, but also considerable differentiation between populations within each region, when summed over all loci (Table 10a,b). Seventy five percent of the polymorphic loci showed significant differences between the populations. These results are confirmed by the *F*-statistic analyses (Table 11a). These indicate that although only about 12% of the total genetic variation is attributable to differentiation between populations, as opposed to differences between individuals within populations, this is still significant at the 5% level. Further, about half of this differentiation is due to differences between populations within regions, with the other half due to differences between regions. Both analyses indicate that there is no trend as to which loci and populations contribute to the differentiation between populations, both within and between regions. This randomness suggests that differences in allele frequency between certain populations may be due to genetic drift or possibly very localised, but weak selection. There were only a few alleles that showed any evidence of the frequency following a geographic cline (Fig. 1a), and for the most part, these were only within a particular region. Even these so-called clines may be due to chance, rather than being related to clines in any environmental or other variables. There does appear to be one exception, however, and that is at the *LAP* locus. The switching of the common allele between the three regions is clear (see Fig. 1a). The same allele, LAP^{106}, is common in the West Coast and South Coast regions, whilst LAP^{100} was common in the populations in the Western Overlap region. This difference between the regions is demonstrated very clearly in the *F*-statistics (Table 11a). Over 36% of the total genetic variance at this locus was due to differences between populations, and of this, about 90% was due to the differentiation between regions, with only a small proportion of the total variance attributable to differences between populations within regions. Again, both genetic drift and/or selection may account for these results. If drift is the major factor, then the fact that different alleles are common in different regions would imply reduced gene flow between the populations in the different regions. If this was the case, however, one might expect to find similar patterns in at least some of the other polymorphic loci. There is a change in the common allele between the West Coast and Western Overlap regions at the *ODH* locus,

although it is not as distinct as that for *LAP*. It is also possible that there are different selective forces operating in the different regions, either directly on the *LAP* locus itself, or on a closely linked locus or group of loci, which maintain the frequency differences between the regions. Deviations of genotypic frequencies from those expected from Hardy-Weinberg proportions can provide indirect evidence in support of selection. Significant deviations were found for *LAP* at three of the *B. cincta* populations (Table 3), and at a further four populations the observed number of heterozygotes was less than the number expected. The greatest changes in environment, particularly sea temperature, probably occur in the relatively small zone between the West Coast and Western Overlap regions (between Kommetjie and Castle Rock sites), so it is here that one might expect to find the greatest influence of selection if there is any, as well as reduced gene flow between populations. In a study of *Perna perna*, Grant *et al.* (1992) found strong allele frequency differences for two loci around Cape Point and suggested that this could be due to temperature selection in the colder waters of the West Coast. In terms of geographic distance, there is a relatively large gap (about 235 km) between the most easterly population in the Western Overlap region, Sparks Bay (SB), and the next population, Mossel Bay (MB), on the South Coast. To determine more precisely the change-over point of the common allele between these two regions, and therefore enable a better assessment of the possible reasons for the differences in allele frequency, a number of populations in between SB and MB would need to be sampled.

Although there appears to be no pattern as to which populations contribute most to the differentiation between populations, both within and between regions, a pattern is evident when Nei's genetic distance, *D*, between all pairs of populations, is plotted against geographic distance (Fig. 7a). The greater the geographic distance between populations, the larger the genetic distance between them; the relationship is significant ($N=45$ pairs of populations, $r=0.59$, $p<0.001$). This 'smoothing out' of the apparently random differences between populations, is expected, since *D* is based on all of the loci examined. Therefore, whilst certain polymorphic loci may act as markers for particular populations, or groups of populations, the overall pattern indicates that the more geographically separated two populations are, the more genetically different they are. This raises the

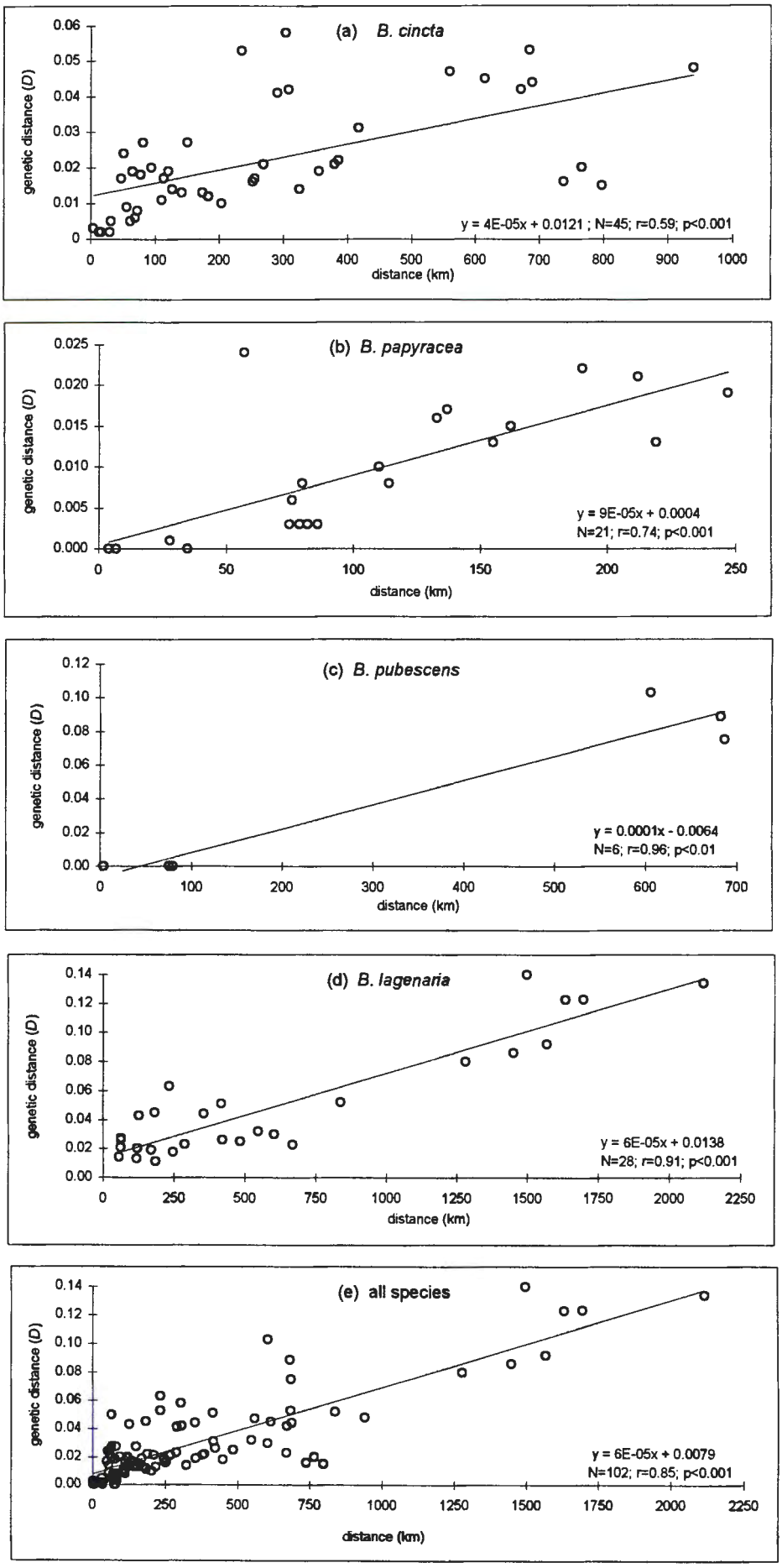


Figure 7. Nei's genetic distance (*D*) as a function of geographic distance between populations for each of the species separately, and all species together.

question as to whether the populations at either end of the range, which would tend to have the greater *D* values, are capable of interbreeding and are therefore, still members of the same species.

The greatest *D* between any two populations of *B. cincta* is 0.058, between A-Frame (AF) and Mossel Bay. The value between the two populations collected at either end of the sampled range, Paternoster and Port Elizabeth, is 0.048. However, even the maximal *D* value of 0.058 fits well within that expected for populations belonging to the same species (Thorpe, 1983; Woodruff *et al.*, 1988), although taxonomic status cannot be based on this criterion alone. In both the phenogram (based on Nei's *I*) and the Wagner network (Figs 4 and 6), all of the *B. cincta* populations group together. This indicates that these populations are more similar, genetically, to each other than they are to any of the other species. This in itself does not imply that populations at either end of the range definitely belong to the same species, but taken together with the very small *D* values between the populations, it must be considered highly unlikely that all of the populations sampled do not belong to the same species.

B. papyracea

As was found with the populations of *B. cincta*, there were significant differences in allele frequency between the populations of *B. papyracea*, although fewer of the polymorphic loci (50% as opposed to 75%) showed significant differences (Table 10a). However, most of the differences were due to differences between regions for some of the loci, or to differences between populations within the Western Overlap region for other loci (Table 10c). The populations from the West Coast were found to be more homogeneous. This species does have a smaller range than that of *B. cincta*, and therefore one might expect there to be less differentiation between the loci when all populations are considered.

The *F*-statistics reflect the reduced levels of differentiation, with the proportion of total variance due to differences between populations being only about seven percent, which was not

significant (Table 11b). About 90% of this differentiation was attributable to differences between populations within regions, with only about 10% due to differences between regions (Fig. 2b). Taken together with the results of the contingency table analysis, it is obvious that it is differences between the populations in the Western Overlap region that contribute most to the differentiation found between these populations. This was mostly due to frequency differences in two populations, Rooiels (RE) and Hermanus (HM), at *AAT-2* and *ARK* respectively.

Similar to *B. cincta*, there was a switch in the common allele between the West Coast and Western Overlap regions (Fig. 1b), in this instance at two of the loci, *ARK* and *GL* (the loci involved with *B. cincta* were *LAP* and *ODH*). These were the only loci where the populations had a different common allele, which reinforces the view that there might be reduced gene flow around Cape Point. Localised selection also cannot be ruled out as a possible factor involved in the change over, although the situation is not as clear-cut as it is in the *B. cincta-LAP* case.

If *LAP* were under direct selection, then one might expect this locus to exhibit a similar pattern in the populations of *B. papyracea* as that shown by *B. cincta*, since it is reasonable to assume similar physiological adaptations to environmental variables in closely related species. However, all of the populations of *B. papyracea* are fixed for LAP^{100}, the allele found in the Western Overlap populations of *B. cincta*.

As with the *B. cincta* populations, there was a significant positive correlation ($N=21$, $r=0.74$, $p<0.001$) between geographic and genetic distance for 21 pairs of populations (Fig. 7b), a reflection that gene flow is greatest between populations in close proximity. The geographic range of *B. papyracea* is much smaller than that of *B. cincta*, and it would be reasonable to expect that the maximum genetic distance between *B. papyracea* populations be lower than that found between populations of *B. cincta*. This is the case, as can be seen in Table 12, where the largest *D* between any two populations is 0.024, less than half that found in *B. cincta*. However, comparisons of genetic distances between populations at equivalent sites in each of these two species, showed that these were very similar. Based on the linear regression equations, the predicted genetic distance

between two populations situated 250 km apart, was the same for both *B. cincta* and *B. papyracea* (D=0.021).

B. pubescens

Three of the four populations of *B. pubescens* were collected in the Western Overlap region, whilst the fourth was collected on the South Coast at Port Elizabeth (PE), which is geographically well separated from the other three sites (between 600 and 700 km). Taking this large distance between the sites into consideration, it is perhaps surprising that there were significant differences in allele frequency in only four of the 15 polymorphic loci in the contingency table analysis (Table 10a), compared with almost twice as many in the *B. papyracea* populations, of which the furthest apart were only 250km. It is possible, with the smaller sample sizes collected for this species, that not all of the significant differences in allele frequency were detected. Such may indeed be the case for three loci (*MDH-1*, *MPI* and *PGM* - see Appendix A). For the four loci that did show significant differences, these differences were considerable, with the PE population having different common alleles (Fig. 1c) to the other populations. At three of these loci, the allele that was common in the Western Overlap populations was not even present in the PE population.

The three Western Overlap populations were fairly homogeneous (Table 10d), but there were big differences between these and the PE population, which are clearly reflected in Wright's *F*-statistics (Table 11c and Fig. 2c). The amount of variability between populations was about 18% of the total variability and, whilst this was not significant, it was still higher than that found in the populations of *B. cincta*. It is obvious that almost all of the differentiation between populations was due to the PE population, as evidenced by the fact that most of this variation between populations was due to variation between regions.

A comparison of Figs 2b and 2c for *B. papyracea* and *B. pubescens* respectively, show completely opposite trends: with *B. papyracea*, there is very little difference between regions, whilst

most of the differences between *B. pubescens* are between regions. This probably reflects the differences in the ranges of the two species. For both, a large portion of the range was sampled, but *B. papyracea* has a much smaller range than *B. pubescens*, and therefore this result would be expected. The collection of additional populations of *B. pubescens* between the PE and Western Overlap sites might alter the composition of Fig. 2c, and a pattern more similar to that found for *B. cincta* in Fig. 2a might be predicted.

As could be expected, the genetic distances between the Western Overlap populations were very low, whilst those between the Western Overlap and PE populations were relatively higher (Table 12), with a mean *D* of 0.089. This is almost twice as high as that between the *B. cincta* South Coast populations (PE and MB) and the Western Overlap populations. Whilst the addition of more sites between PE and the Western Overlap might affect the picture presented by the results of the *F*-statistics for *B. pubescens*, they will not affect the higher genetic distances it exhibits as compared to *B. cincta*.

The relatively high *D* between the PE and the other *B. pubescens* populations (reflected in Figs 4 and 6), again raises the question asked of the *B. cincta* populations: are the populations at either end of the range capable of interbreeding? However, the highest *D* (0.103 between the PE and Rooiels populations) still lies reasonably well within the conspecific population range according to Thorpe's (1982) survey. Furthermore, the locus *MDH-2* is diagnostic for all four populations of *B. pubescens*, and *PGD* is diagnostic for three of the populations, including PE (Table 6). This would imply that the PE population is not, or was not at some stage, genetically isolated from the Western Overlap populations of this species. Thus, there is no evidence to suggest that these populations do not belong to the same species.

From the genetic distances between the *B. pubescens* populations, the correlation between geographic distance and Nei's *D* was, as expected, significant ($N=6$, $r=0.96$, $p<0.01$: Fig. 7c). Although there were only six points on the graph, based on the results of the other species, it is unlikely that this picture would alter greatly if further sites of intermediate geographic distances

were added. What may change however, is the slope of the regression line which was steeper (by an order of magnitude) for *B. pubescens* than the other species. At a geographic distance of 500km, the predicted genetic distance would be 0.065 for *B. pubescens* , as compared with 0.030 for *B. cincta*.

B. lagenaria

The results for this species are very similar to those for *B. cincta*, in that not only were there significant differences in allele frequency between all populations, but also between populations within the regions (Table 10a,e). The *F*-statistics reflect a similar pattern, with almost equal amounts of the variation attributable to differences between populations within regions, and between regions (compare Figs 2a for *B. cincta* and 2d for *B. lagenaria*). This similarity is probably due to the fact that for both species, most of the range of the species was sampled, with relatively large numbers of populations being sampled throughout the range. There is a big difference, however, in the amount of the total genetic variance that is due to differences between populations, as opposed to the total variance within the populations. For *B. cincta*, the former was about 12%, whilst in *B. lagenaria*, it was about 38%, the highest for any of the species. Also, over 90% of the polymorphic loci showed significant allele frequency heterogeneity for *B. lagenaria*, whilst for *B. cincta* this was about 75%. This occurs in spite of the fact that earlier it was noted (page 122) that *B. lagenaria* is one of the species exhibiting the lowest amounts of total genetic variation (Table 4). The amount of inter-population variation due to differences between populations (F_{PT}) is expressed as a proportion of the total variance (H_T) although this total amount differs between the species. What this does indicate is that only a few of the populations were responsible for the differences observed, although different populations could be involved for different loci. At six of the 13 polymorphic loci with significant allele frequency heterogeneity, there was just a single population (not always the same one) that differed from the others, and at another two loci, just two of the populations were different.

As with *B. cincta*, there were five loci where not all of the populations had the same common allele (Fig. 1d). Like both *B. cincta* and *B. papyracea*, there was a switch over of the common allele between West Coast and Western Overlap regions, although only at one of the loci, that of *ODH*. As mentioned in the results, *B. lagenaria* and *B. cincta* share the same common alleles for most of the populations in the different regions. This similarity between the two species may be indicative of some form of selection operating on this locus. If this is the case however, then the selective force does not appear to be that strong, at least in the Western Overlap and South Coast regions. Both species were collected at a number of sites. At Dalebrook (DK), both species had the same common allele, whilst at Sparks Bay (SB), still in the Western Overlap, the common allele was different. Likewise at Mossel Bay (MB) on the South Coast, the two species had different common alleles.

In the Durban (DN) population on the East Coast, *ARK* was fixed for a different allele from the other populations, although this allele was found in the Blouberg (BB) population on the West Coast but at a very low frequency (0.033). Apart from the possibility that only one allele was detected at DN as a result of the smaller sample sizes, this finding indicates that there is no gene flow between the DN population and the others. There are also other loci, most notably *IDH-1* and *PGM*, where the DN population differs substantially from the other populations. This difference is best demonstrated if this population is excluded from the *F*-statistics analysis. The differentiation between populations (F_{PT}) drops from 38% to 26%, indicating that a large portion (almost a third) of this variance is entirely due to the DN population. However, even without this population, the difference between populations was still significant ($p<0.01$). Exclusion of the DN population reduces the F_{PT} for *ARK* from about 96% to almost zero, since most of the other populations were fixed for the same allele. The observation that there is apparently no gene flow between the DN population and the others, does not imply that this population is reproductively isolated from the other populations, since this population is widely separated geographically from the others. The total distance between the collection localities was approximately 2120 km, of which the distance between DN and MB, the nearest neighbouring site, was about 1280 km. Samples collected closer to the DN population would need to be studied to determine just how isolated this population is. It

is unlikely, however, to be completely isolated since the correlation coefficient between geographic and genetic distance is highly significant ($N=28$, $r=0.91$, $p<0.001$, Fig. 7d). In a study of *Goniobasis proxima*, Dillon (1984) found that at six out of seven loci tested, no alleles were shared between two widely separated pops, yet they still constituted a recognisable unit below the generic level. He concluded that without the knowledge that a full range of intergrades existed between the two populations, their conspecific status would be questionable.

As with the other species so far discussed, the genetic distances between the *B. lagenaria* populations are well within the range expected for conspecific populations (Table 12), even when the DN population is considered. Without this population, the highest *D* between any two populations was 0.063. With the DN population included, the highest *D* was 0.140, but according to Thorpe's (1982) survey, 98% of the *D* values between conspecifics are less than 0.16. The big difference between the DN population and the others is clearly reflected in both the phenogram of Nei's *D* (Fig. 4), and the Wagner network based on Rogers' *D* (Fig. 6). The major difference between these two figures is the placement of this DN population. In the phenogram, it is joined to the other *B. lagenaria* populations only after these have been linked to the *B. cincta* populations, whilst in the network, the DN population is clustered with the other *B. lagenaria* populations. This difference reflects not only the difference in the methodology of the two analyses, but also the close similarity between these two species.

B. sp. A

Although only two populations of this species were sampled, it is still possible to draw some conclusions about the population structure of this species. The contingency table analysis indicated significant allele frequency heterogeneity between the populations at five of the nine polymorphic loci and, whilst the *F*-statistics did not detect a significant differentiation between the populations over all loci combined, there was a significant differentiation at the *GL* locus. Both of the populations were collected in the West Coast region, but are separated by a distance of about

450 km, so some differences are expected. As with the other species, most of the total genetic variation was contained within the populations, with about **11%** due to differences between the populations, a proportion similar to that observed in *B. cincta*, *B. papyracea* and *B. pubescens*. The genetic distance of 0.018 between the two populations was low, and is similar to values found between populations from the same region in the other species.

B. catarrhacta

As with *B.* sp. A, only two populations of this species were collected, one on the West Coast and the other in the Western Overlap region. Almost 29% of the total genetic variability was attributable to differences between these populations, a relatively high proportion more similar to that found for *B. lagenaria* than for the other species. There were only four polymorphic loci, and all had a different common allele in the two populations. This observation further reinforces the view that there is either reduced gene flow around Cape Point, or that different selective forces are operating in the two regions. The genetic distance, $D = 0.050$, between the two population is well within the range expected for conspecifics, and is similar to values in the other species found between populations from different regions.

General conclusions about intraspecific differentiation

From the foregoing sections, it is clear that within all of the species there is differentiation between populations. However, it must be borne in mind that this differentiation between populations is relatively low (between 7% and 38% of the total variability) when compared to levels of variation within populations (62% to 93%). This is true even for *B. lagenaria*, for which the amount of differentiation between the populations (38%) was at least twice that of the other species except for *B. catarrhacta*. Janson and Ward (1984) showed that in **11** populations of *Littorina*

saxatilis sampled over a 1km stretch of coastline, only about 7% of the total genetic variation was due to differences between populations. Similar results were found for populations of *Littorina saxatilis* and *L. arcana* with only 12% of the total variability due to population differentiation (Ward & Janson, 1985). Grant and Utter (1987) found that about 33% of the total genetic diversity of *Nucella lamellosa* was due to differentiation between populations. They noted that this appeared to be the greatest amount of population differentiation reported in marine gastropods. In this context, *B. lagenaria* appears to be even more genetically fragmented than *N. lamellosa*.

The loci which contributed to most of the differentiation between populations were not the same for all species, with nine showing significant differentiation, although two of these, *LAP* and *AAT-2*, were significant in three of the species. Hoagland (1984) found similar results in a study of *Crepidula*, and noted that this implied that microevolution of allozymes could proceed quite differently in pairs of congeners living in habitats with similar general ecological requirements. That molecular divergence does not occur at the same rate at all loci for a given set of taxa, has also been discussed by Avise (1976).

In the four species for which more than two populations were sampled from at least two regions, the results indicated differences in the population structure (Fig. 2). For *B. cincta* and *B. lagenaria*, the differentiation between populations was due, more or less equally, to differences between populations within regions, and between regions. For *B. papyracea*, almost all of the differentiation between the populations was due to differences within regions, whilst for *B. pubescens*, almost all population differentiation occurred between regions. In a study of 21 populations of *Nucella lapillus* (of the same karyotype) Day *et al.* (1993) found that differentiation between regions was six times greater than that within regions. Grant and Utter (1987) obtained similar results for *Nucella lamellosa* as those obtained for *B. cincta* and *B. lagenaria* - differences between populations within regions were similar to differences between regions.

The existence of population differentiation would appear to indicate that there is a reduced level of gene flow between certain populations. This situation would be expected when one

considers that *Burnupena* lays eggs on the substratum, and that the young hatch as crawlaways, there being no larval dispersal stage (Bokenham et *al.*, 1938). The species noted above, namely *Nucella lapillus*, *N. lamellosa*, *Littorina saxatilis* and *L. arcana*, that have similar levels of population differentiation to species of *Burnupena*, all lack a planktonic larval stage. Janson (1987) found that levels of population differentiation were higher in *Littorina saxatilis* than in a co-occurring species, *L. littorea*, which has a planktonic larval stage (the proportion of total diversity due to differentiation between populations was 8% and 2% respectively). *Nucella lapillus* showed considerable interpopulational heterogeneity on two different geographic scales (over several 100km - Day & Bayne, 1988, and within 21km - Day, 1990). Gastropods which have a planktonic larval stage on the other hand, tend to have lower levels of population differentiation. Gooch et *al.* (1972) found no differences in allele frequency for six loci in 11 populations of *Nassarius obsoletus* sampled over 1000km. Two genetically distinct groups of *Stramonita haemostoma*, a thaidid mollusc with a planktonic larval stage, both showed little geographic variation across distances of 1500km (Liu et *al.*, 1991). However, whilst marine invertebrates without pelagic larvae do tend to show greater population differentiation than species having planktonic larvae, substantial differentiation has been observed in species that appear to have high dispersal capabilities (Burton, 1983; Hedgecock, 1986), and caution must therefore be exercised when inferring levels of gene flow from apparent dispersal capabilities. Little is known about the extent of dispersal by adult *Burnupena*, but other studies of dog whelks indicate that the adults are rather sedentary (Hughes, 1972 in Day & Bayne, 1988). Even if the snails were fairly mobile, stretches of sandy beach interspersed between the rocky shores would limit dispersal. Under these conditions the relatively large amounts of genetic differentiation observed between populations was not unexpected. However, in a study on drift-dispersal of molluscs with direct development, Martel and Chia (1991) showed that postmetamorphic drifting was a common feature of the life history of many marine bivalves and gastropods, regardless of their mode of larval development. Along with many other species, they collected newly emerged *Nucella emarginata* in their drift collectors, and suggested they too were capable of drifting. They suggest that drifting may be one of the factors responsible for the wide geographic distributions of many species which lack a planktonic larval stage.

For the four species sampled at more than two sites, there was a significant correlation between genetic and geographic distances (Fig. 7a-d). Likewise, when all species were combined (Fig. 7e), the correlation was highly significant ($N = 102$ pairs of conspecific populations, $r = 0.85$, $p < 0.001$). This pattern is consistent with an isolation-by-distance (Wright, 1943) model of the genetic structure of the species. That is, although any species consists of a continuous population, the individuals within the species only exchange genes with individuals that are closest to them geographically. This type of genetic structure is consistent with the lack of a larval dispersal phase. Janson (1987) found a strong positive correlation between genetic and geographic distances for *Littorina saxatilis*, which has direct development, but no correlation in *L. littorea*, which has a planktonic larval stage. A positive correlation between these two parameters was also found for populations of *Haliotis rubra* (Brown, 1991), although it practices external fertilization. However, larval dispersal is thought to be restricted in this species (Prince et *al.*, 1987), and localised genetic heterogeneity has been recorded (Brown, 1991). Palmer et *al.* (1990) note that reproductive isolation between distant populations could reflect either isolation-by-distance, or the existence of two species. In his study on *Nucella emarginata*, reproductive and morphological differences supported high genetic distances, which lead to his conclusion that two species exist.

In my study, the zoogeographic regions used were those put forward by Stephenson (1944). These are essentially the same as those proposed by Emanuel et *al.* (1992), excepting that they combined the Western Overlap and South Coast regions. These two regions were analysed separately due to the large geographic distance between the sites that were sampled within each of them (approximately 170km between closest sites in the two regions, as opposed to about 50 km between the closest two populations from the West Coast and Western Overlap regions). For the most part, the conspecific populations within a region clustered together (Fig. 4), the Hermanus populations of *B. papyracea* and *B. lagenaria* and the *B. papyracea* Rooiels population being the exceptions. The results also indicated that the Western Overlap populations were genetically more similar to the West Coast populations than those of the South Coast, even although there were changes in the common allele around Cape Point in all four species sampled from both sides of Cape Point, involving eight loci.

Whether the changes in the common allele in populations on either side of Cape Point were due to reduced gene flow and subsequent genetic drift, or to differential selection, are not clear. Although selection cannot be ruled out, since there are differences in sea temperature between the two regions, it seems unlikely, due to the fact that most of the loci involved were unique to particular species, only one recurring in more than one species.

Nei's *D* ranged from zero to 0.140 between conspecific populations. This range is similar to that recorded for other species of whelks. In a study of four populations of *Nucella lapillus* (Day & Bayne, 1988), the genetic distance ranged from 0.003 to 0.019. In a subsequent study of 21 populations from across the range of this species (separated by up to 10 000km), Day et *al.* (1993) recorded genetic distances of between zero and 0.076, with only a slight increase in *D* with geographic distance. The mean *D* between populations separated by less than 200km was 0.009, whilst that between populations separated by more than 200km was 0.027. Genetic distances between widely separated populations of *Nucella emarginata* ranged from zero to 0.187 (Palmer et *al.*, 1990 - excluding the southern population, suspected of being a cryptic species) The largest distances (0.145 to 0.187) were between a geographically widely separated population and the remaining populations (over 1000km apart). These genetic distances were similar to those obtained for the *B. pubescens* Port Elizabeth and *B. lagenaria* Durban populations, which displayed the highest genetic differentiation from other populations of their respective species. They were both geographically widely separated from the remaining populations sampled. If populations were to be collected at sites nearer to these two populations, they may prove less distinct. However, the relatively high *D* values between these two populations and the others will not be altered.

Comparisons of Nei's identity values for conspecific populations revealed results similar to those found by Thorpe (1982). About 85% of the *I* values were greater than 0.95, with 96% above 0.90 and 100% above 0.85 (Thorpe's percentages were 80%, 93% and 98% respectively). He noted that if there are two allopatric populations of dubious status, and *I* is less than 0.85, then it would

be improbable that they would be conspecific. In this study none of the conspecific comparisons were below 0.87 (Fig. 3).

Both the Wagner network and the UPGMA phenogram revealed that all of the populations of the same species cluster together, with the single exception of the *B. lagenaria* Durban population, which was not clustered together with the other *B. lagenaria* populations in the UPGMA phenogram. There is however, no justification for separating the Durban population, since the inclusion of other samples from the East Coast could alter the linkage of this population in the phenogram. The fact that conspecific populations, in some cases widely separated geographically, clustered together, supports the initial starting classification. Genetic distance or similarity values cannot be used on their own to define cut-off points between taxa, and can at best only be used as a guideline. However, they can be a useful when breeding studies cannot be made, to confirm the existence, or otherwise, of specific status.

Interspecific genetic differentiation and systematics

Having examined the levels of differentiation within the species of *Burnupena*, it is now possible to consider the amount of differentiation that exists between species. For all species, there was significant differentiation between populations within a species. Taking this into consideration, the results clearly indicted that, for the most part, the species were well differentiated.

Over 40% of the alleles, occurring in 18 loci, were specific to one species. Almost one half of these were found only in *B. catarrhacta*, about one quarter in *B. pubescens*, whilst the remaining quarter were spread between the other species. However, apart from most of the alleles specific to *B. catarrhacta*, many of these species-specific alleles were rare and/or were present in only a few of the populations sampled.

Table 15. Key classifying individuals of *Burnupena* from any population, using the presence or absence of alleles. The numbers in parentheses are that percentage of individuals out of the total number of individuals screened, in all populations, that are expected to be classified at that level.

1. *PGD*$^{-655}$ detected.....	*B. catarrhacta*	(100%)
PGD^{-655} not detected.....	2	
2. *MDH-2*^{36} detected.....	*B. pubescens*	(100%)
MDH-2^{36} not detected.....	3	
3. *PGD*$^{-455}$ detected.....	*B. papyracea*	(100%)
PGD^{-455} not detected.....	4	
4. *PGD*$^{-300}$ detected.....	*B*. sp. A	(100%)
PGD^{-300} not detected.....	5	
5. *PGD*$^{-490}$ detected.....	*B. lagenaria*	(96%)
PGD^{-490} not detected.....	6	
6. *PGD*$^{-260}$ detected.....	*B*. sp. B	(29%)
PGD^{-260} not detected.....	7	
7. *LAP*^{103} detected.....	*B*. sp. B	(100%)
LAP^{103} not detected.....	8	
8. *LAP*^{100} or *LAP*^{106} detected,	{99.8% chance that it is *B. cincta*;	
LAP^{109} not detected.....	{0.2% chance that it is *B. lagenaria*.	
LAP^{109} detected,	{91% chance that it is *B. lagenaria*;	
LAP^{100} and *LAP*^{106} not detected...	{9% chance that it is *B. cincta*.	

As a result of the high proportion of species-specific alleles, seventeen of the polymorphic loci were diagnostic for *B. catarrhacta*. In eighteen of the 34 populations sampled, at least 99% of the individuals could be assigned to the correct species using a single locus. In particular, *PGD* was diagnostic for 16 of these. All ten *B. cincta* populations, together with five from *B. lagenaria*, and the one *B.* sp. B population, had no diagnostic loci when compared with all other populations. However, pairwise comparisons between species indicated that only between *B. cincta* and *B. lagenaria* was there no individual diagnostic locus. These two species could be distinguished, however, by considering two loci jointly. As expected, over 68% of loci were diagnostic for *B. catarrhacta*, with between 16% and 20% diagnostic for *B. pubescens*. The remaining species had 12% or fewer diagnostic loci (apart from comparisons with *B. catarrhacta* and *B. pubescens*). In a review of studies using electrophoretic techniques, Avise (1975) found that most closely related species were almost completely distinct in allelic composition at an average of 25% to 50% of their loci.

Virtually all of the populations sampled can be identified to specific level by following a key based on a combination of loci and species-specific alleles (see Table 15). This type of biochemical key has been used before (Avise, 1975) to classify species. The key uses the probability of an individual having a particular allele, based on allele frequencies calculated from data pooled from all of the populations in each species. The percentage of individuals expected to be classified at a particular level is also based on the pooled data. For example, PGD^{-490} is specific for *B. lagenaria*, so any individual having this allele can be assigned to this species, even if the individual is a heterozygote and the other allele occurs in other species. Using allele frequencies from the pooled data, the frequency of individuals homozygous for PGD^{-490} was 0.65, and the frequency of heterozygous individuals with this allele was 0.31. Hence, this allele would be detected in 96% of the individuals. Since the allele frequencies are calculated from the pooled data, for particular populations, the percentage of individuals expected to be keyed out at that level may not be entirely accurate depending on how the allele frequencies of that population differ from those for the species as a whole. This would explain the apparent discrepancy between the fact that 96% of

the *B. lagenaria* individuals can be classified using *PGD*, yet, as was seen in Table 6, this locus was diagnostic for only three of the eight populations tested. Five populations were not diagnosable using PGD^{-490}. Nevertheless, the number of individuals in these populations that could be keyed out using the presence of PGD^{-490} was between 81% and 98%. This was however, not enough to be 99% certain of making the correct species assignment, which was the criterion for a diagnostic locus.

As noted, apart from *B. catarrhacta*, only one other allele was fixed in all populations of a particular species, namely $MDH\text{-}2^{36}$ in *B. pubescens*. Following the key, all individuals belonging to *B. catarrhacta* and *B. pubescens* could be classified by their possession of PGD^{-655} and $MDH\text{-}2^{36}$ alleles respectively. Next, a series of alleles for *PGD* were used to distinguish all *B. papyracea* and all *B.* sp. A individuals, most of the *B. lagenaria* (96%), and 29% of the *B.* sp. B individuals. Not all of the individuals could be separated in the latter two species, since these alleles were not fixed in all populations. The remainder of individuals belonging to *B.* sp. B (about 71%) were then identified using LAP^{103}. Both this species and the two *B.* sp. A populations were fixed for LAP^{103}, but since all of the *B.* sp. A individuals should have already been keyed out by their possession of PGD^{-300} (couplet 4), any individual still unclassified that has LAP^{103} should, in theory, belong to *B.* sp. B. Any individuals which have not been identified when couplet 8 is reached, should either belong to *B. cincta* or *B. lagenaria*, with the majority belonging to *B. cincta* since 96% of the individuals belonging to *B. lagenaria* would already have been identified (couplet 5). It is not possible to unambiguously separate all individuals belonging to these two species, but the probability of making the correct assignment will be high.

It must be borne in mind that this key works for the populations that have so far been examined, but there may be alleles which have so far not been found in some species which may later be discovered. Conversely, alleles which are used in the key to identify some species may not be present in other populations of that species.

The results of the *F*-statistics showed that the loci that contributed the most to the differentiation between the populations were not always the same as those in the within-species analyses. Although the loci did not all contribute equally, they did, however, all show significant differences between populations. Of the total variation, 68% was attributable to differentiation between populations, which was almost twice as high as the maximum found within a species (38% in *B. lagenaria*). Over two thirds of the differentiation between populations was due to differences between species and only about a third due to differences between populations within species. This puts the levels of differentiation between populations within a species clearly into perspective. In a study of 50 populations of three closely related species of the *Littorina saxatilis* species complex, Knight and Ward (1991) found that only about 27% of the total variation was due to differences between populations, and that the proportion attributable to species differentiation was similar to that due to differentiation among populations within species. Exclusion of *B. catarrhacta* from the analysis reduced the proportion of differentiation between populations to 60%, but did not alter the overall picture. At some loci, *B. catarrhacta* is well differentiated, whereas there is no differentiation between the rest of the species at these loci. However, there are many other loci that do differentiate between the other species.

Nei's *D* between species ranged from 0.054 to 1.583, although the maximum *D* was reduced to 0.450 if *B. catarrhacta* was excluded. The lowest interspecific *D* values were between populations of *B. cincta* and *B. lagenaria*, as expected, since there was no single locus diagnostic between these two species. This was clearly shown in Fig. 3, as were the high *D* values between *B. catarrhacta* and the other species. In a review of studies on *Littorina* species (Ward, 1990), genetic distances between non-sibling species pairs ranged from 0.137 to 2.38. Palmer *et al.* (1990) found evidence for the existence of two cryptic species of *Nucella emarginata*, with genetic distances between them ranging from 0.162 to 0.313.

The phenogram (Fig. 4) further indicated that each of the species was a distinct entity, with the exception of the Durban *B. lagenaria* population, which was, on average, more different from its conspecifics than these were to *B. cincta*. Although there was no diagnostic locus between these

two species, there was one locus (*ARK*) that was diagnostic for Durban *B. lagenaria*. The Wagner network includes this population within the *B. lagenaria* cluster.

In his survey, Thorpe (1982) found that only about 3% of I values between congenerics were greater than 0.85, and only 5% were above 0.80. In this study, 21% of the comparisons between species were greater that 0.85, almost all of these between *B. cincta* and *B. lagenaria*. About 40% of the I values were above 0.80. However, these results are similar to those suggested by Woodruff *et al.* (1988) for sexually reproducing molluscs, where most of the congeneric distances (D) were greater than 0.05 (I =0.95), and usually between 0.20 and 0.60 (I = 0.82 and 0.55 respectively - see Fig. 3).

As noted above, genetic distance or similarity values cannot be used to define cut-off points between taxa. From the cut-off lines corresponding to I values of 0.85, 0.90 and 0.95 shown on the phenogram, it is clear that no one line would generate the original seven taxa, even if the Durban population is ignored. For *B. cincta* and *B. lagenaria* to form separate clusters, a 0.95 cut-off would be needed, but then the *B. pubescens* populations would be split. A cut-off of 0.90 would avoid this, but then *B. cincta* and *B. lagenaria* would be united. Clearly, *B. cincta* and *B. lagenaria* are genetically distinct, although they do exhibit much lower levels of differentiation relative to the other species, as well as overlapping the range of differentiation between conspecifics. It must be remembered that enzyme electrophoresis underestimates the differences between taxa, and therefore *B. cincta* and *B. lagenaria* are likely to be more different than is apparent. At the other end of the scale, *B. catarrhacta* is very well differentiated from the other species. The I values fall into the middle of the range of values found between genera, although there is a very big overlap in I between congenerics and different genera, and the *B. catarrhacta* values still fall within the range of congeneric differences (Fig. 4e in Thorpe, 1983). The I and D values are just a guideline, and are usually more concerned with differences between conspecifics and congeners.

During this study a new species, referred to as *B.* sp. B, was detected. Although individuals were initially identified as either *B. pubescens* or *B. papyracea*, the results of the enzyme

electrophoresis showed that this species was clearly genetically distinct from both of them. At least 4% of the loci were diagnostic, and Nei's mean *D* values between *B.* sp. B and *B. papyracea* or *B. pubescens* were 0.194 and 0.309 respectively ($I<0.83$). Electrophoresis has been used for the detection of a number of morphologically cryptic species (Chambers, 1978; Mastro *et al.*, 1982; Janson, 1985; Ward & Janson, 1985; Staub *et al.*, 1990).

A lack of gene flow between two sympatric populations provides a much stronger case for reproductive isolation than does genetic distance. In the 18 comparisons made between sympatric populations (*B. catarrhacta* was excluded), all except one showed at least one locus where no alleles were shared by the two species, implying reproductive isolation between the two particular species. The single exception was between *B. cincta* and *B. lagenaria* at Dalebrook. However, there was no genotypic overlap at one locus, which also indicates an absence of gene flow between these two populations. A comparison of the *D* values between the sympatric populations, revealed that in all but the *B. cincta* - *B. lagenaria* comparisons, the *D* values between the sympatric populations were at least twice that of the maximum *D* found between the conspecific populations of the two species concerned. For *B. cincta* and *B. lagenaria*, the maximum *D* within each species (0.058 and 0.140 respectively) were similar to the *D*'s between the sympatric populations (between 0.061 and 0.128 for the four localities). Comparisons between *B.* sp. B and both *B. papyracea* and *B. pubescens* showed that no alleles were shared at at least two loci, leaving no doubt that reproductive isolation is total and that we are dealing with a new species. The implication of this analysis is that, apart from *B. cincta* and *B. lagenaria* comparisons, populations of different species which occur in sympatry are more differentiated than are populations of the same species that are well separated geographically.

This study was initially undertaken because there were problems with the identification of some of the species belonging to *Burnupena*. The results of the enzyme electrophoresis have indicated that the current nominate morphologically-based species do in fact represent genetically distinct entities, despite the difficulties of separating some populations morphologically. In addition,

the electrophoretic approach unearthed a previously unrecognised cryptic species which almost certainly would not have been detected by a purely morphological approach.

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Appendix A. Allele frequencies for 22 polymorphic loci in 34 populations of *Burnupena* .
N = number of individuals examined.

Locus	*B. cincta*										*B. papyracea*						
Allele	PT	BB	OK	KM	CR	AF	DK	SB	MB	PE	BB	BO	LL	CR	AF	RE	HM
ARK																	
100	-	-	-	-	-	-	-	-	-	-	0.591	0.522	0.500	0.393	0.473	0.483	1.000
86	-	-	-	-	-	-	-	-	-	-	0.152	0.300	0.300	0.595	0.514	0.517	-
82	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
70	0.600	0.167	0.217	0.321	0.260	0.375	0.268	0.375	0.040	-	-	0.033	-	0.012	0.014	-	-
52	0.400	0.833	0.783	0.679	0.740	0.625	0.732	0.625	0.960	1.000	0.258	0.144	0.200	-	-	-	-
32	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N	30	18	30	28	25	48	71	24	50	5	33	45	5	42	37	29	3
AAT-1																	
-50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-61	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-65	-	-	-	-	-	-	-	-	-	-	0.015	-	-	-	-	-	-
-100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.985	1.000	1.000	1.000	1.000	1.000	1.000
N	30	18	30	28	25	48	71	32	50	5	33	45	5	42	37	29	3
AAT-2																	
127	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
100	0.759	0.917	0.950	0.804	0.940	0.976	0.965	0.719	0.680	1.000	0.955	0.933	0.800	0.905	0.859	0.500	1.000
65	0.241	0.083	0.050	0.196	0.060	0.024	0.035	0.281	0.320	-	0.045	0.067	0.200	0.095	0.141	0.500	-
N	29	18	30	28	25	42	71	32	50	2	33	45	5	42	32	22	3
DIA-1																	
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.621	0.689	0.600	0.643	0.541	0.615	0.833
94	-	-	-	-	-	-	-	-	-	-	0.379	0.311	0.400	0.357	0.459	0.385	0.167
N	30	18	30	28	25	48	71	32	50	2	33	45	5	42	37	26	3
G6PDH																	
104	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
N	30	18	30	28	25	48	71	32	50	5	33	45	5	42	37	29	3
GPI																	
575	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
450	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
315	-	-	-	-	0.020	0.031	-	-	-	-	0.015	-	-	0.012	-	-	-
200	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
100	1.000	1.000	1.000	1.000	0.980	0.969	1.000	1.000	1.000	1.000	0.985	1.000	1.000	0.988	0.986	1.000	1.000
-86	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.014	-	-
-150	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-165	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N	30	18	30	28	25	48	71	32	50	5	33	45	5	42	37	29	3
GAP																	
-100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
-450	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-520	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N	30	18	30	27	25	48	71	29	50	3	33	42	5	42	37	29	3
HEX-3																	
101	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.944	0.800	0.976	1.000	1.000	1.000
98	-	-	-	-	-	-	-	-	-	-	-	0.056	0.200	0.012	-	-	-
97	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
95	-	-	-	-	-	-	-	-	-	-	-	-	-	0.012	-	-	-
92	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N	30	18	30	28	25	48	71	32	50	5	33	45	5	42	37	29	3
IDH-1																	
236	-	-	0.017	-	0.100	0.010	0.077	0.016	0.316	0.200	-	0.012	-	0.071	0.029	0.167	-
100	1.000	1.000	0.983	1.000	0.900	0.990	0.923	0.984	0.663	0.800	1.000	0.977	1.000	0.929	0.971	0.833	1.000
10	-	-	-	-	-	-	-	-	0.020	-	-	0.012	-	-	-	-	-
N	30	18	30	28	25	48	71	32	49	5	32	43	5	42	35	27	3

(cont.)

Appendix A (continued)

Locus	*B. cincta*										*B. papyracea*						
Allele	PT	BB	OK	KM	CR	AF	DK	SB	MB	PE	BB	BO	LL	CR	AF	RE	HM
IDH-2																	
117	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
100	1.000	1.000	1.000	1.000	1.000	1.000	0.993	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
83	-	-	-	-	-	-	0.007	-	-	-	-	-	-	-	-	-	-
N	30	18	30	28	25	47	71	32	50	5	33	45	5	42	37	29	3
LAP																	
109	0.217	0.028	0.200	0.107	0.020	-	0.007	0.031	-	-	-	-	-	-	-	-	-
106	0.467	0.639	0.733	0.768	0.240	0.156	0.275	0.234	1.000	1.000	-	-	-	-	-	-	-
103	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
100	0.317	0.333	0.067	0.125	0.740	0.844	0.718	0.734	-	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000
90	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N	30	18	30	28	25	48	71	32	50	5	33	45	5	42	37	29	3
MDH-1																	
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.967	1.000	0.917	0.959	1.000	1.000
-32	-	-	-	-	-	-	-	-	-	-	-	0.033	-	0.083	0.041	-	-
-65	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-280	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N	30	18	30	28	25	48	71	32	50	5	32	45	5	42	37	29	3
MDH-2																	
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
36	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-67	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N	30	18	30	28	25	48	71	32	50	2	33	45	5	42	37	29	3
MDH-3																	
100	0.983	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
95	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
82	0.017	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N	30	18	30	28	25	48	71	32	50	5	33	45	5	42	37	29	3
ME-3																	
102	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
N	30	18	30	28	25	48	71	32	40	5	33	45	5	42	37	29	3
MPI																	
106	0.317	0.028	0.100	0.054	-	0.229	0.127	0.141	0.050	-	-	0.011	-	-	-	0.034	-
100	-	-	-	-	-	-	-	-	-	-	1.000	0.989	1.000	1.000	1.000	0.966	1.000
97	0.617	0.778	0.600	0.554	0.740	0.573	0.585	0.625	0.340	0.500	-	-	-	-	-	-	-
88	0.050	0.194	0.300	0.393	0.260	0.198	0.289	0.234	0.610	0.500	-	-	-	-	-	-	-
78	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
75	0.017	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N	30	18	30	28	25	48	71	32	50	5	33	45	5	42	37	29	3
ODH																	
125	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
115	-	0.056	-	-	-	-	-	-	-	-	0.078	0.170	0.200	0.179	0.109	0.056	0.333
100	0.380	0.333	0.370	0.423	0.520	0.560	0.602	0.339	0.459	0.250	0.922	0.807	0.800	0.810	0.891	0.944	0.667
85	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
82	0.520	0.583	0.500	0.558	0.340	0.393	0.266	0.571	0.163	0.750	-	-	-	-	-	-	-
69	-	-	-	-	-	-	-	-	-	-	-	0.023	-	0.012	-	-	-
62	0.100	0.028	0.130	0.019	0.140	0.048	0.133	0.089	0.378	-	-	-	-	-	-	-	-
N	25	18	27	26	25	42	64	28	49	4	32	44	5	42	32	27	3
GL																	
129	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
119	-	-	-	-	-	-	-	-	0.160	-	-	-	-	-	-	-	-
115	0.717	0.722	0.733	0.857	0.840	0.917	0.972	0.922	0.790	1.000	-	-	-	-	-	-	-
111	-	-	-	-	-	-	-	-	-	-	0.273	0.378	0.200	0.537	0.541	0.672	0.833
100	0.283	0.278	0.267	0.143	0.160	0.083	0.028	0.078	0.050	-	0.712	0.622	0.800	0.463	0.459	0.328	0.167
91	-	-	-	-	-	-	-	-	-	-	0.015	-	-	-	-	-	-
N	30	18	30	28	25	48	71	32	50	4	33	45	5	41	37	29	3

(cont.)

Appendix A (continued)

Locus	*B. cincta*										*B. papyracea*						
Allele	PT	BB	OK	KM	CR	AF	DK	SB	MB	PE	BB	BO	LL	CR	AF	RE	HM
PHP																	
104	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
95	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
87	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
81	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N	30	18	30	28	25	48	71	32	50	5	33	45	5	42	37	29	3
PGM																	
121	-	-	-	-	-	-	-	-	-	-	-	-	-	0.036	0.014	0.017	-
111	0.017	0.056	-	-	-	0.063	0.042	0.042	-	-	0.197	0.178	0.200	0.143	0.108	0.103	0.167
100	0.533	0.472	0.367	0.518	0.560	0.469	0.387	0.604	0.490	0.400	0.803	0.811	0.800	0.810	0.878	0.879	0.833
86	0.350	0.278	0.517	0.339	0.360	0.365	0.437	0.292	0.280	-	-	0.011	-	-	-	-	-
69	0.100	0.194	0.117	0.143	0.080	0.104	0.134	0.063	0.230	0.600	-	-	-	0.012	-	-	-
N	30	18	30	28	25	48	71	24	50	5	33	45	5	42	37	29	3
PGD																	
800	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
455	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	-	-	-	-	-	0.167
-182	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-260	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-300	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-455	-	-	-	-	-	-	-	-	-	-	1.000	1.000	1.000	1.000	1.000	1.000	0.833
-490	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-655	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N	30	18	28	28	25	48	70	31	47	5	33	45	5	42	37	29	3
SDH																	
118	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
106	-	0.194	-	-	-	0.107	0.134	0.313	-	0.200	0.136	0.067	-	-	-	-	-
100	1.000	0.806	1.000	1.000	1.000	0.893	0.866	0.688	1.000	0.800	0.864	0.933	1.000	1.000	1.000	1.000	1.000
77	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N	30	18	30	28	25	42	67	32	10	5	33	45	5	42	32	29	3

(cont.)

Appendix A (continued)

Locus	*B. lagenaria*								*B. pubescens*				*B.* sp. A		*B. catarrhacta*		*B.* sp. B
Allele	GR	BB	KM	DK	SB	HM	MB	DN	CR	AF	RE	PE	GR	BO	KM	DK	AF
ARK																	
100	-	-	-	-	-	-	-	-	-	-	-	-	-	0.011	-	-	-
86	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
82	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.000	1.000	-
70	-	0.033	-	-	-	-	-	1.000	0.576	0.536	0.714	-	-	0.011	-	-	0.684
52	1.000	0.967	1.000	1.000	1.000	1.000	1.000	-	0.394	0.446	0.286	0.800	1.000	0.978	-	-	0.316
32	-	-	-	-	-	-	-	-	0.030	0.018	-	0.200	-	-	-	-	-
N	20	15	22	25	15	17	40	10	33	28	7	10	23	45	20	27	19
AAT-1																	
-50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.000	1.000	-
-61	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.053
-65	-	-	-	-	-	-	-	-	0.030	-	-	-	-	-	-	-	-
-100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.970	1.000	1.000	1.000	1.000	1.000	-	-	0.947
N	20	15	22	25	15	17	40	10	33	28	7	10	23	45	20	27	19
AAT-2																	
127	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.800	-	-
100	1.000	1.000	1.000	0.980	0.767	1.000	1.000	1.000	0.970	1.000	1.000	1.000	0.761	0.378	0.200	1.000	-
65	-	-	-	0.020	0.233	-	-	-	0.030	-	-	-	0.239	0.622	-	-	1.000
N	20	15	22	25	15	17	40	10	33	22	7	10	23	45	20	27	19
DIA-1																	
100	0.450	0.700	1.000	1.000	1.000	1.000	1.000	1.000	-	-	-	-	-	-	-	-	0.026
94	0.550	0.300	-	-	-	-	-	-	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.974
N	20	15	22	25	15	17	40	10	33	28	7	10	23	45	20	27	19
G6PDH																	
104	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.000	1.000	-
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	-	1.000
N	20	15	22	25	15	17	40	10	33	28	7	10	23	45	20	27	19
GPI																	
575	-	-	-	-	-	-	-	-	-	-	-	0.050	-	-	-	-	-
450	-	-	-	-	-	-	-	-	0.136	0.036	0.071	0.650	-	-	-	-	-
315	-	-	-	-	-	-	-	-	0.470	0.500	0.500	0.150	-	-	-	-	0.026
200	-	-	-	-	-	-	-	-	0.121	0.268	0.286	-	-	-	-	-	-
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.258	0.196	0.143	0.150	1.000	1.000	-	-	0.974
-86	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-150	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.000	1.000	-
-165	-	-	-	-	-	-	-	-	0.015	-	-	-	-	-	-	-	-
N	20	15	22	25	15	17	40	10	33	28	7	10	23	45	20	27	19
GAP																	
-100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	-	1.000
-450	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.632	0.241	-
-520	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.368	0.759	-
N	20	15	15	24	15	17	39	2	33	27	6	5	23	44	19	27	19
HEX-3																	
101	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.600	0.333	-
100	0.625	1.000	1.000	1.000	1.000	0.971	1.000	1.000	0.955	0.982	1.000	1.000	0.978	1.000	-	-	1.000
98	-	-	-	-	-	0.029	-	-	-	-	-	-	0.022	-	-	-	-
97	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.400	0.667	-
95	-	-	-	-	-	-	-	-	0.045	0.018	-	-	-	-	-	-	-
92	0.375	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N	20	15	22	25	15	17	40	10	33	28	7	10	23	45	20	27	19
IDH-1																	
236	-	-	-	-	-	-	-	0.600	-	0.023	-	-	0.848	0.782	-	-	-
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.400	1.000	0.977	1.000	1.000	0.152	0.218	1.000	1.000	1.000
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N	20	15	22	25	15	17	37	10	33	22	7	10	23	39	20	27	19

(cont.)

Appendix A (continued)

Locus	*B. lagenaria*								*B. pubescens*				*B.* sp. A		*B. catarrhacta*		*B.* sp. B
Allele	GR	BB	KM	DK	SB	HM	MB	DN	CR	AF	RE	PE	GR	BO	KM	DK	AF
IDH-2																	
117	-	-	0.045	-	-	-	-	-	0.015	-	-	-	-	-	-	-	-
100	1.000	0.733	0.909	1.000	1.000	0.971	0.887	1.000	0.985	0.964	0.929	1.000	1.000	1.000	1.000	1.000	0.974
83	-	0.267	0.045	-	-	0.029	0.112	-	-	0.036	0.071	-	-	-	-	-	0.026
N	20	15	22	25	15	17	40	10	33	28	7	10	23	45	20	27	19
LAP																	
109	1.000	0.533	1.000	1.000	1.000	1.000	1.000	1.000	0.167	0.161	0.143	1.000	-	-	-	-	-
106	-	0.433	-	-	-	-	-	-	0.742	0.804	0.857	-	-	-	-	-	-
103	-	-	-	-	-	-	-	-	-	-	-	-	1.000	1.000	-	-	1.000
100	-	0.033	-	-	-	-	-	-	0.091	0.036	-	-	-	-	-	-	-
90	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.000	1.000	-
N	20	15	22	25	15	17	40	10	33	28	7	10	23	45	20	27	18
MDH-1																	
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.182	0.107	0.143	0.333	1.000	1.000	-	-	0.763
-32	-	-	-	-	-	-	-	-	0.818	0.893	0.857	0.667	-	-	-	-	-
-65	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.237
-280	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.000	1.000	-
N	20	15	22	25	15	17	40	10	33	28	7	9	23	45	20	27	19
MDH-2																	
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	-	-	-	1.000	1.000	-	-	1.000
36	-	-	-	-	-	-	-	-	1.000	1.000	1.000	1.000	-	-	-	-	-
-67	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.000	1.000	-
N	20	15	22	25	15	17	40	10	33	28	7	10	23	45	20	27	19
MDH-3																	
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	-	1.000
95	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.000	1.000	-
82	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N	20	15	22	25	15	17	40	10	33	28	7	10	23	45	20	27	19
ME-3																	
102	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.000	1.000	-
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	-	1.000
N	20	15	22	25	15	17	39	10	33	28	7	10	23	43	20	27	19
MPI																	
106	-	-	0.023	-	-	-	-	0.200	-	-	-	-	-	-	-	-	0.132
100	-	-	-	0.042	-	-	-	-	-	-	-	-	-	-	-	-	0.474
97	1.000	1.000	0.977	0.958	1.000	1.000	0.938	0.800	0.333	0.286	0.286	0.500	-	0.089	-	-	-
88	-	-	-	-	-	-	-	-	-	-	-	-	1.000	0.911	-	-	0.395
78	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.000	1.000	-
75	-	-	-	-	-	-	0.063	-	0.667	0.714	0.714	0.500	-	-	-	-	-
N	20	15	22	24	15	17	40	10	33	28	7	10	23	45	20	27	19
ODH																	
125	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.000	1.000	-
115	-	-	-	-	-	-	-	-	0.016	-	-	-	-	-	-	-	-
100	0.469	0.133	0.375	0.609	0.889	0.156	0.077	-	0.922	0.929	0.929	1.000	1.000	1.000	-	-	0.947
85	-	-	-	-	-	-	-	-	0.063	0.071	0.071	-	-	-	-	-	-
82	0.531	0.867	0.625	0.391	0.111	0.844	0.923	1.000	-	-	-	-	-	-	-	-	-
69	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
62	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.053
N	16	15	20	23	9	16	39	10	32	28	7	10	23	45	20	27	19
GL																	
129	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.000	1.000	-
119	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
115	0.900	0.867	0.550	1.000	0.900	0.941	0.850	1.000	-	-	-	-	0.348	-	-	-	-
111	-	-	-	-	-	-	-	-	1.000	1.000	1.000	1.000	0.435	1.000	-	-	0.974
100	0.100	0.133	0.450	-	0.100	0.059	0.150	-	-	-	-	-	0.217	-	-	-	0.026
91	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N	20	15	20	25	15	17	40	10	33	28	7	10	23	40	20	27	19

(cont.)

Appendix A (continued)

Locus	*B. lagenaria*								*B. pubescens*				*B.* sp. A		*B. catarrhacta*		*B.* sp. B
Allele	GR	BB	KM	DK	SB	HM	MB	DN	CR	AF	RE	PE	GR	BO	KM	DK	AF
PHP																	
104	-	0.200	-	0.020	-	-	-	-	0.045	-	-	0.050	-	-	-	-	-
100	1.000	0.800	1.000	0.980	1.000	1.000	1.000	1.000	0.955	1.000	1.000	0.950	0.978	0.833	-	-	1.000
95	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.000	1.000	-
87	-	-	-	-	-	-	-	-	-	-	-	-	0.022	0.156	-	-	-
81	-	-	-	-	-	-	-	-	-	-	-	-	-	0.011	-	-	-
N	20	15	22	25	15	17	40	10	33	28	7	10	23	45	20	27	19
PGM																	
121	-	-	-	-	-	-	-	-	0.697	0.804	0.857	0.950	-	-	-	-	-
111	-	-	-	-	-	-	-	-	0.303	0.196	0.143	0.050	0.152	0.022	-	-	0.053
100	0.825	0.967	1.000	0.480	0.967	0.706	-	0.050	-	-	-	-	0.848	0.978	-	-	0.947
86	0.175	-	-	0.240	0.033	0.118	0.675	-	-	-	-	-	-	-	0.775	0.204	-
69	-	0.033	-	0.280	-	0.176	0.325	0.950	-	-	-	-	-	-	0.225	0.796	-
N	20	15	22	25	15	17	40	10	33	28	7	10	23	45	20	27	19
PGD																	
800	-	-	-	-	-	-	-	-	-	-	-	0.050	-	-	-	-	-
455	-	-	-	-	-	-	-	-	0.297	0.140	0.071	0.950	-	-	-	-	-
-100	-	-	-	0.440	0.233	0.147	0.287	0.400	-	0.060	-	-	-	-	-	-	0.816
-182	-	-	-	-	-	-	-	-	0.703	0.800	0.929	-	-	-	-	-	0.026
-260	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.158
-300	-	-	-	-	-	-	-	-	-	-	-	-	0.978	0.944	-	-	-
-455	-	-	-	-	-	-	-	-	-	-	-	-	0.022	0.056	-	-	-
-490	1.000	1.000	1.000	0.560	0.767	0.853	0.712	0.600	-	-	-	-	-	-	-	-	-
-655	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.000	1.000	-
N	20	15	20	25	15	17	40	5	32	25	7	10	23	45	20	27	19
SDH																	
118	-	-	-	-	-	-	-	-	-	0.045	0.143	-	-	-	-	-	-
106	0.025	-	-	0.020	-	-	0.038	-	0.197	0.114	0.143	0.250	-	-	-	-	-
100	0.975	1.000	1.000	0.980	1.000	1.000	0.962	1.000	0.803	0.841	0.714	0.750	1.000	1.000	-	-	1.000
77	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.000	1.000	-
N	20	15	22	25	15	17	40	10	33	22	7	10	23	44	20	27	19

Appendix B. Observed heterozygosities for each of the polymorphic loci in each of the 34 populations of *Burnupena*. *ME-3*, *G6PDH* and *MDH-2* have been excluded because none of the populations were polymorphic for these loci.

	locus																		
population	*ARK*	*AAT-1*	*AAT-2*	*DIA-1*	*GPI*	*GAP*	*HEX-3*	*IDH-1*	*IDH-2*	*LAP*	*MDH-1*	*MDH-3*	*MPI*	*ODH*	*GL*	*PHP*	*PGM*	*PGD*	*SDH*
PAP-BB	0.667	0.030	0.091	0.515	0.030	0	0	0	0	0	0	0	0	0.156	0.455	0	0.273	0	0.212
PAP-BO	0.556	0	0.133	0.400	0	0	0.111	0.047	0	0	0.067	0	0.022	0.227	0.400	0	0.244	0	0.089
PAP-LL	0.200	0	0.400	0.800	0	0	0.400	0	0	0	0	0	0	0.400	0.400	0	0.400	0	0
PAP-CR	0.524	0	0.190	0.524	0.024	0	0.048	0.143	0	0	0.167	0	0	0.238	0.488	0	0.286	0	0
PAP-AF	0.514	0	0.281	0.432	0.027	0	0	0.057	0	0	0.081	0	0	0.094	0.432	0	0.216	0	0
PAP-RE	0.276	0	0.273	0.462	0	0	0	0.259	0	0	0	0	0.069	0.037	0.448	0	0.172	0	0
PAP-HM	0	0	0	0.333	0	0	0	0	0	0	0	0	0	0.667	0.333	0	0.333	0.333	0
B-AF	0.526	0.105	0	0.053	0.053	0	0	0	0.053	0	0.263	0	0.526	0.105	0.053	0	0.105	0.368	0
PUB-CR	0.485	0.061	0.061	0	0.606	0	0.091	0	0.030	0.212	0.364	0	0.485	0.156	0	0.091	0.303	0.469	0.394
PUB-AF	0.643	0	0	0	0.643	0	0.036	0.045	0.071	0.179	0.143	0	0.286	0.143	0	0	0.393	0.320	0.227
PUB-RE	0.286	0	0	0	0.857	0	0	0	0.143	0.286	0.286	0	0.286	0.143	0	0	0.286	0.143	0.286
PUB-PE	0.200	0	0	0	0.500	0	0	0	0	0	0.222	0	0.400	0	0	0.100	0.100	0.100	0.100
LIM-PT	0.467	0	0.345	0	0	0	0	0	0	0.433	0	0.033	0.433	0.400	0.367	0	0.533	0	0
LIM-BB	0.222	0	0.056	0	0	0	0	0	0	0.389	0	0	0.333	0.5	0.556	0	0.611	0	0.167
LIM-OK	0.433	0	0.100	0	0	0	0	0.033	0	0.200	0	0	0.567	0.481	0.467	0	0.567	0	0
LIM-KM	0.429	0	0.393	0	0	0	0	0	0	0.250	0	0	0.500	0.346	0.286	0	0.500	0	0
CIN-CR	0.520	0	0.120	0	0.040	0	0	0.200	0	0.440	0	0	0.360	0.480	0.320	0	0.600	0	0
CIN-AF	0.458	0	0.048	0	0.063	0	0	0.021	0	0.188	0	0	0.521	0.286	0.125	0	0.521	0	0.119
CIN-DK	0.310	0	0.07	0	0	0	0	0.155	0.014	0.254	0	0	0.563	0.438	0.056	0	0.549	0	0.209
CIN-SB	0.667	0	0.375	0	0	0	0	0.031	0	0.281	0	0	0.500	0.571	0.156	0	0.417	0	0.313
CIN-MB	0.080	0	0.52	0	0	0	0	0.531	0	0	0	0	0.500	0.469	0.340	0	0.640	0	0
CIN-PE	0	0	0	0	0	0	0	0	0	0	0	0	0.200	0.500	0	0	0.400	0	0
A-GR	0	0	0.391	0	0	0	0.043	0.217	0	0	0	0	0	0	0.261	0.043	0.304	0.043	0
A-BO	0.044	0	0.356	0	0	0	0	0.385	0	0	0	0	0.044	0	0	0.289	0.044	0.111	0
LAG-GR	0	0	0	0.400	0	0	0.350	0	0	0	0	0	0	0.188	0.200	0	0.350	0	0.050
LAG-BB	0.067	0	0	0.333	0	0	0	0	0.400	0.333	0	0	0	0.133	0.267	0.267	0.067	0	0
LAG-KM	0	0	0	0	0	0	0	0	0.182	0	0	0	0.045	0.450	0.500	0	0	0	0
LAG-DK	0	0	0.040	0	0	0	0	0	0	0	0	0	0	0.348	0	0.040	0.880	0.400	0.040
LAG-SB	0	0	0.333	0	0	0	0	0	0	0	0	0	0	0.222	0.200	0	0.067	0.467	0
LAG-HM	0	0	0	0	0	0	0.059	0	0.059	0	0	0	0	0.188	0.118	0	0.412	0.294	0
LAG-MB	0	0	0	0	0	0	0	0	0.225	0	0	0	0.075	0.154	0.200	0	0.400	0.375	0.075
LAG-DN	0	0	0	0	0	0	0	0.600	0	0	0	0	0.400	0	0	0	0.100	0	0
CAT-KM	0	0	0.300	0	0	0.316	0.600	0	0	0	0	0	0	0	0	0	0.350	0	0
CAT-DK	0	0	0	0	0	0.259	0.593	0	0	0	0	0	0	0	0	0	0.407	0	0

Appendix C. Matrix of genetic similarity and distance between all 34 populations of *Burnupena*. Below diagonal:Nei's (1978) genetic distance *D*. Above diagonal: Nei's genetic identity *I*.

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 CIN-PE	*****	0.980	0.956	0.959	0.949	0.957	0.985	0.981	0.985	0.953	0.789	0.762	0.771	0.776	0.783	0.787	0.787
2 CIN-MB	0.021	*****	0.949	0.959	0.943	0.959	0.981	0.979	0.970	0.954	0.803	0.795	0.792	0.796	0.805	0.804	0.803
3 CIN-SB	0.045	0.053	*****	0.991	0.994	0.992	0.981	0.973	0.988	0.986	0.848	0.833	0.835	0.838	0.847	0.849	0.847
4 CIN-DK	0.042	0.041	0.009	*****	0.998	0.998	0.981	0.980	0.986	0.980	0.853	0.831	0.839	0.843	0.849	0.853	0.852
5 CIN-AF	0.053	0.058	0.006	0.002	*****	0.997	0.976	0.974	0.983	0.984	0.862	0.839	0.849	0.853	0.859	0.863	0.861
6 CIN-CR	0.044	0.042	0.008	0.002	0.003	*****	0.983	0.982	0.989	0.984	0.859	0.840	0.848	0.851	0.860	0.861	0.859
7 LIM-KM	0.016	0.019	0.019	0.019	0.024	0.017	*****	0.998	0.995	0.990	0.829	0.812	0.817	0.820	0.829	0.830	0.827
8 LIM-OK	0.020	0.022	0.027	0.020	0.027	0.018	0.002	*****	0.995	0.987	0.825	0.802	0.812	0.817	0.827	0.828	0.827
9 LIM-BB	0.015	0.031	0.012	0.014	0.017	0.011	0.005	0.005	*****	0.987	0.836	0.813	0.823	0.827	0.839	0.840	0.840
10 LIM-PT	0.048	0.047	0.014	0.021	0.017	0.016	0.010	0.013	0.013	*****	0.846	0.832	0.836	0.840	0.849	0.849	0.844
11 PAP-HM	0.237	0.220	0.165	0.159	0.148	0.152	0.188	0.192	0.179	0.167	*****	0.976	0.984	0.983	0.980	0.987	0.982
12 PAP-RE	0.272	0.229	0.182	0.185	0.175	0.175	0.209	0.221	0.207	0.184	0.024	*****	0.994	0.992	0.987	0.985	0.979
13 PAP-AF	0.260	0.233	0.180	0.176	0.164	0.165	0.202	0.208	0.195	0.179	0.016	0.006	*****	1.000	0.998	0.997	0.992
14 PAP-CR	0.253	0.229	0.176	0.171	0.159	0.161	0.198	0.202	0.190	0.175	0.017	0.008	0.000	*****	0.997	0.997	0.990
15 PAP-LL	0.245	0.217	0.166	0.163	0.152	0.151	0.187	0.190	0.175	0.164	0.021	0.013	0.003	0.003	*****	1.000	1.000
16 PAP-BO	0.240	0.218	0.164	0.159	0.148	0.149	0.187	0.189	0.174	0.164	0.013	0.015	0.003	0.003	0.000	*****	0.999
17 PAP-BB	0.240	0.220	0.166	0.160	0.150	0.151	0.190	0.190	0.174	0.169	0.019	0.022	0.008	0.010	0.000	0.001	*****
18 PUB-PE	0.397	0.395	0.385	0.359	0.364	0.359	0.368	0.351	0.358	0.367	0.362	0.365	0.346	0.351	0.369	0.358	0.353
19 PUB-RE	0.385	0.377	0.390	0.368	0.369	0.372	0.350	0.348	0.358	0.349	0.364	0.370	0.349	0.352	0.380	0.365	0.365
20 PUB-AF	0.365	0.355	0.375	0.349	0.352	0.352	0.334	0.330	0.340	0.338	0.350	0.356	0.335	0.338	0.363	0.350	0.350
21 PUB-CR	0.357	0.349	0.356	0.334	0.335	0.337	0.323	0.321	0.327	0.323	0.330	0.334	0.315	0.318	0.341	0.329	0.329
22 LAG-DN	0.127	0.175	0.129	0.137	0.132	0.135	0.124	0.126	0.130	0.100	0.297	0.324	0.317	0.305	0.313	0.303	0.315
23 LAG-MB	0.094	0.128	0.094	0.091	0.095	0.088	0.083	0.068	0.073	0.085	0.266	0.301	0.284	0.276	0.262	0.260	0.262
24 LAG-HM	0.093	0.126	0.089	0.096	0.096	0.085	0.083	0.078	0.075	0.085	0.237	0.266	0.251	0.246	0.234	0.232	0.235
25 LAG-SB	0.118	0.111	0.092	0.088	0.092	0.078	0.085	0.085	0.084	0.086	0.208	0.212	0.209	0.209	0.195	0.196	0.195
26 LAG-DK	0.077	0.093	0.070	0.061	0.066	0.057	0.061	0.054	0.055	0.064	0.214	0.238	0.226	0.222	0.212	0.209	0.209
27 LAG-KM	0.127	0.144	0.111	0.118	0.116	0.100	0.103	0.096	0.092	0.099	0.216	0.235	0.219	0.216	0.198	0.199	0.198
28 LAG-BB	0.094	0.125	0.100	0.110	0.110	0.096	0.086	0.086	0.078	0.095	0.234	0.254	0.236	0.235	0.222	0.223	0.223
29 LAG-GR	0.139	0.157	0.124	0.124	0.126	0.113	0.117	0.111	0.110	0.119	0.243	0.252	0.233	0.236	0.218	0.223	0.220
30 A-BO	0.282	0.224	0.278	0.269	0.282	0.259	0.262	0.274	0.279	0.284	0.261	0.212	0.236	0.246	0.250	0.254	0.252
31 A-GR	0.233	0.192	0.246	0.224	0.238	0.219	0.226	0.232	0.239	0.253	0.263	0.227	0.231	0.239	0.232	0.238	0.231
32 CAT-DK	1.312	1.409	1.358	1.325	1.320	1.344	1.340	1.317	1.312	1.340	1.337	1.396	1.307	1.315	1.307	1.311	1.301
33 CAT-KM	1.502	1.495	1.427	1.418	1.418	1.438	1.422	1.397	1.420	1.411	1.466	1.458	1.412	1.430	1.405	1.427	1.418
34 B-AF	0.278	0.216	0.208	0.226	0.222	0.218	0.205	0.229	0.232	0.191	0.204	0.159	0.183	0.197	0.198	0.206	0.210

(cont.)

Appendix C (continued)

Population	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
1 CIN-PE	0.672	0.680	0.694	0.700	0.881	0.910	0.911	0.889	0.926	0.881	0.911	0.870	0.754	0.792	0.269	0.223	0.757
2 CIN-MB	0.674	0.686	0.701	0.706	0.840	0.880	0.882	0.895	0.911	0.866	0.882	0.855	0.799	0.826	0.244	0.224	0.806
3 CIN-SB	0.681	0.677	0.687	0.701	0.879	0.910	0.915	0.912	0.933	0.895	0.905	0.883	0.757	0.782	0.257	0.240	0.812
4 CIN-DK	0.699	0.692	0.705	0.716	0.872	0.913	0.909	0.916	0.941	0.889	0.896	0.884	0.764	0.799	0.266	0.242	0.798
5 CIN-AF	0.695	0.691	0.703	0.715	0.877	0.909	0.909	0.912	0.936	0.891	0.896	0.882	0.754	0.788	0.267	0.242	0.801
6 CIN-CR	0.699	0.689	0.704	0.714	0.874	0.916	0.919	0.925	0.945	0.905	0.909	0.893	0.772	0.803	0.261	0.237	0.804
7 LIM-KM	0.692	0.705	0.716	0.724	0.883	0.920	0.921	0.918	0.941	0.902	0.918	0.890	0.770	0.798	0.262	0.241	0.815
8 LIM-OK	0.704	0.706	0.719	0.726	0.882	0.935	0.925	0.918	0.947	0.908	0.918	0.895	0.760	0.793	0.268	0.247	0.796
9 LIM-BB	0.699	0.699	0.712	0.721	0.878	0.930	0.928	0.919	0.946	0.912	0.925	0.896	0.757	0.787	0.269	0.242	0.793
10 LIM-PT	0.693	0.705	0.713	0.724	0.905	0.919	0.919	0.918	0.938	0.905	0.909	0.888	0.753	0.777	0.262	0.244	0.826
11 PAP-HM	0.696	0.695	0.705	0.719	0.743	0.766	0.789	0.812	0.808	0.806	0.791	0.785	0.770	0.769	0.263	0.231	0.815
12 PAP-RE	0.694	0.691	0.700	0.716	0.724	0.740	0.767	0.809	0.788	0.791	0.775	0.777	0.809	0.797	0.248	0.233	0.853
13 PAP-AF	0.707	0.706	0.715	0.730	0.728	0.753	0.778	0.811	0.798	0.803	0.789	0.792	0.790	0.793	0.271	0.244	0.833
14 PAP-CR	0.704	0.704	0.713	0.728	0.737	0.759	0.782	0.811	0.801	0.806	0.790	0.790	0.782	0.788	0.269	0.239	0.821
15 PAP-LL	0.691	0.684	0.696	0.711	0.731	0.769	0.791	0.823	0.809	0.820	0.801	0.804	0.779	0.793	0.271	0.245	0.820
16 PAP-BO	0.699	0.694	0.705	0.720	0.739	0.771	0.793	0.822	0.811	0.819	0.800	0.800	0.776	0.788	0.270	0.240	0.814
17 PAP-BB	0.703	0.694	0.705	0.720	0.730	0.770	0.791	0.823	0.812	0.820	0.800	0.802	0.777	0.794	0.272	0.242	0.811
18 PUB-PE	*****	0.902	0.915	0.928	0.653	0.718	0.718	0.742	0.747	0.726	0.709	0.754	0.716	0.713	0.303	0.271	0.717
19 PUB-RE	0.103	*****	1.000	1.000	0.638	0.649	0.648	0.670	0.675	0.656	0.674	0.682	0.690	0.687	0.304	0.272	0.732
20 PUB-AF	0.089	0.000	*****	1.000	0.640	0.665	0.664	0.686	0.692	0.670	0.687	0.698	0.706	0.704	0.306	0.273	0.739
21 PUB-CR	0.075	0.000	0.000	*****	0.652	0.675	0.674	0.697	0.702	0.681	0.697	0.710	0.716	0.713	0.311	0.279	0.751
22 LAG-DN	0.427	0.450	0.446	0.427	*****	0.923	0.918	0.870	0.912	0.884	0.884	0.874	0.676	0.712	0.260	0.205	0.699
23 LAG-MB	0.331	0.432	0.408	0.393	0.080	*****	0.982	0.939	0.977	0.957	0.951	0.949	0.700	0.734	0.266	0.243	0.702
24 LAG-HM	0.331	0.433	0.410	0.394	0.086	0.019	*****	0.974	0.987	0.989	0.982	0.977	0.731	0.761	0.258	0.225	0.726
25 LAG-SB	0.298	0.400	0.377	0.361	0.140	0.063	0.026	*****	0.987	0.980	0.956	0.970	0.777	0.798	0.242	0.220	0.782
26 LAG-DK	0.292	0.393	0.369	0.354	0.092	0.023	0.013	0.014	*****	0.973	0.958	0.969	0.751	0.782	0.266	0.234	0.758
27 LAG-KM	0.321	0.422	0.400	0.384	0.123	0.044	0.011	0.020	0.027	*****	0.980	0.976	0.750	0.777	0.248	0.217	0.741
28 LAG-BB	0.344	0.395	0.376	0.361	0.123	0.051	0.018	0.045	0.043	0.021	*****	0.974	0.754	0.781	0.261	0.228	0.744
29 LAG-GR	0.282	0.382	0.360	0.343	0.134	0.052	0.023	0.030	0.032	0.025	0.026	*****	0.771	0.801	0.281	0.254	0.759
30 A-BO	0.334	0.371	0.348	0.334	0.392	0.356	0.314	0.252	0.286	0.287	0.283	0.260	*****	0.982	0.236	0.226	0.901
31 A-GR	0.338	0.375	0.351	0.339	0.339	0.309	0.273	0.226	0.245	0.253	0.247	0.222	0.018	*****	0.251	0.227	0.863
32 CAT-DK	1.195	1.189	1.186	1.168	1.347	1.323	1.356	1.417	1.324	1.394	1.345	1.268	1.443	1.383	*****	0.951	0.255
33 CAT-KM	1.305	1.301	1.298	1.276	1.583	1.413	1.491	1.515	1.452	1.529	1.479	1.370	1.488	1.481	0.050	*****	0.258
34 B-AF	0.333	0.312	0.303	0.286	0.358	0.354	0.320	0.246	0.277	0.300	0.296	0.275	0.105	0.147	1.365	1.354	*****

Appendix D. Matrix of Rogers' modified distance (Wright, 1978) between all 34 populations.

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 CIN-PE	*****																
2 CIN-MB	0.154	*****															
3 CIN-SB	0.212	0.216	*****														
4 CIN-DK	0.204	0.192	0.096	*****													
5 CIN-AF	0.227	0.226	0.085	0.051	*****												
6 CIN-CR	0.211	0.196	0.098	0.058	0.065	*****											
7 LIM-KM	0.141	0.135	0.138	0.135	0.150	0.131	*****										
8 LIM-OK	0.152	0.143	0.160	0.138	0.157	0.135	0.063	*****									
9 LIM-BB	0.142	0.170	0.116	0.121	0.132	0.111	0.086	0.083	*****								
10 LIM-PT	0.218	0.203	0.121	0.139	0.127	0.129	0.104	0.116	0.121	*****							
11 PAP-HM	0.453	0.430	0.379	0.374	0.363	0.367	0.402	0.406	0.394	0.380	*****						
12 PAP-RE	0.469	0.427	0.385	0.389	0.380	0.380	0.411	0.422	0.410	0.386	0.171	*****					
13 PAP-AF	0.462	0.432	0.384	0.380	0.369	0.372	0.406	0.411	0.400	0.382	0.148	0.084	*****				
14 PAP-CR	0.455	0.427	0.379	0.374	0.363	0.366	0.401	0.405	0.393	0.377	0.150	0.096	0.036	*****			
15 PAP-LL	0.453	0.422	0.374	0.373	0.362	0.362	0.396	0.399	0.385	0.372	0.176	0.135	0.095	0.099	*****		
16 PAP-BO	0.445	0.418	0.366	0.362	0.351	0.353	0.391	0.392	0.379	0.366	0.136	0.121	0.065	0.064	0.059	*****	
17 PAP-BB	0.446	0.421	0.370	0.365	0.355	0.357	0.395	0.396	0.381	0.373	0.154	0.145	0.093	0.103	0.070	0.048	*****
18 PUB-PE	0.551	0.541	0.533	0.520	0.524	0.521	0.526	0.516	0.520	0.522	0.534	0.527	0.517	0.518	0.532	0.522	0.521
19 PUB-RE	0.542	0.529	0.534	0.523	0.525	0.527	0.513	0.513	0.518	0.510	0.534	0.528	0.517	0.516	0.536	0.524	0.526
20 PUB-AF	0.527	0.512	0.521	0.508	0.511	0.511	0.500	0.498	0.503	0.499	0.522	0.516	0.505	0.504	0.523	0.512	0.514
21 PUB-CR	0.519	0.504	0.506	0.495	0.497	0.499	0.489	0.488	0.491	0.486	0.506	0.499	0.488	0.488	0.506	0.495	0.497
22 LAG-DN	0.343	0.386	0.336	0.346	0.340	0.344	0.331	0.334	0.338	0.299	0.498	0.507	0.504	0.494	0.503	0.492	0.503
23 LAG-MB	0.297	0.332	0.288	0.284	0.290	0.280	0.272	0.247	0.257	0.274	0.473	0.489	0.478	0.471	0.465	0.459	0.462
24 LAG-HM	0.298	0.332	0.283	0.292	0.292	0.277	0.274	0.266	0.262	0.277	0.453	0.466	0.456	0.450	0.446	0.439	0.442
25 LAG-SB	0.331	0.314	0.287	0.281	0.288	0.268	0.279	0.279	0.277	0.279	0.428	0.422	0.421	0.419	0.412	0.408	0.408
26 LAG-DK	0.271	0.286	0.250	0.234	0.244	0.229	0.235	0.223	0.227	0.240	0.430	0.441	0.432	0.427	0.424	0.416	0.417
27 LAG-KM	0.341	0.352	0.313	0.322	0.319	0.300	0.303	0.294	0.289	0.298	0.435	0.442	0.429	0.425	0.414	0.410	0.411
28 LAG-BB	0.296	0.328	0.295	0.308	0.308	0.290	0.276	0.276	0.265	0.288	0.446	0.453	0.439	0.437	0.431	0.427	0.428
29 LAG-GR	0.353	0.364	0.327	0.326	0.329	0.314	0.319	0.312	0.311	0.320	0.454	0.452	0.438	0.438	0.428	0.428	0.427
30 A-BO	0.482	0.429	0.470	0.464	0.475	0.458	0.460	0.469	0.472	0.474	0.470	0.420	0.442	0.449	0.456	0.455	0.455
31 A-GR	0.444	0.399	0.445	0.428	0.440	0.425	0.431	0.436	0.441	0.450	0.471	0.433	0.438	0.442	0.441	0.442	0.437
32 CAT-DK	0.828	0.832	0.821	0.820	0.821	0.825	0.823	0.820	0.818	0.818	0.836	0.835	0.824	0.823	0.822	0.822	0.823
33 CAT-KM	0.850	0.839	0.826	0.829	0.830	0.833	0.830	0.827	0.828	0.823	0.850	0.839	0.835	0.835	0.831	0.834	0.836
34 B-AF	0.476	0.418	0.411	0.427	0.424	0.422	0.410	0.430	0.433	0.395	0.420	0.367	0.392	0.404	0.410	0.412	0.417

(cont.)

Appendix D (continued)

Population	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
18 PUB-PE	*****																
19 PUB-RE	0.307	*****															
20 PUB-AF	0.281	0.051	*****														
21 PUB-CR	0.259	0.079	0.054	*****													
22 LAG-DN	0.569	0.579	0.574	0.560	*****												
23 LAG-MB	0.511	0.567	0.551	0.539	0.274	*****											
24 LAG-HM	0.514	0.571	0.555	0.543	0.284	0.136	*****										
25 LAG-SB	0.492	0.554	0.537	0.524	0.355	0.242	0.162	*****									
26 LAG-DK	0.484	0.545	0.528	0.516	0.291	0.149	0.116	0.120	*****								
27 LAG-KM	0.507	0.565	0.550	0.537	0.335	0.205	0.108	0.142	0.163	*****							
28 LAG-BB	0.517	0.544	0.530	0.517	0.333	0.218	0.137	0.209	0.202	0.146	*****						
29 LAG-GR	0.476	0.539	0.522	0.508	0.346	0.221	0.152	0.173	0.175	0.157	0.162	*****					
30 A-BO	0.514	0.535	0.518	0.506	0.555	0.531	0.506	0.461	0.483	0.487	0.479	0.463	*****				
31 A-GR	0.516	0.536	0.518	0.507	0.522	0.499	0.476	0.438	0.451	0.460	0.451	0.431	0.133	*****			
32 CAT-DK	0.805	0.800	0.796	0.787	0.839	0.833	0.841	0.850	0.831	0.847	0.831	0.821	0.851	0.841	*****		
33 CAT-KM	0.819	0.813	0.810	0.801	0.865	0.841	0.855	0.859	0.845	0.860	0.844	0.832	0.853	0.850	0.218	*****	
34 B-AF	0.510	0.494	0.484	0.470	0.531	0.526	0.507	0.453	0.474	0.493	0.485	0.471	0.306	0.357	0.834	0.828	*****

Chapter 4

Review of the genus Burnupena Iredale, 1918 (Gastropoda: Buccinidae), with descriptions of two new species

INTRODUCTION

The members of the genus *Burnupena* have had a complex taxonomic history, with the generic name changing from *Buccinum* or *Purpura*, then to *Cominella*, before the genus *Burnupena* was erected by Iredale in 1918. Only three of the original specific names have been retained, namely, *papyracea*, *lagenaria* and *limbosa*. The other species have had to be renamed, either because their names had been used earlier to describe a completely different species, or because an earlier name had precedence.

Notwithstanding two relatively recent revisions of the genus (Orr, 1956; Barnard, 1959), difficulties in identification remain, mainly due to the occurrence of phenotypic intermediates between sympatric forms (Kilburn, 1972, Kilburn & Rippey, 1982). Orr (1956) recognised only two species, *B. papyracea* (Bruguière,1789) and *B. delalandii* (Kiener, 1834 - now *B. catarrhacta* (Gmelin, 1791)). She also recognised four subspecies of *B. papyracea*, namely *B. p. papyracea* (Bruguière,1789), *B. p. cincta* (Röding, 1798), *B. p. lagenaria* (Lamarck, 1822) and *B. p. tigrina* (Kiener, 1834 - now *B. pubescens* (Küster, 1858)). Barnard (1959) on the other hand, recognised six species, elevating Orr's four subspecies, as well as one of her synonyms of *B. papyracea*, namely *B. limbosa* (Lamarck, 1822), to specific level, although he noted that apart from the taxonomic status, there was little difference between his and Orr's conclusions.

Orr and Barnard's differences of opinion about the *B. papyracea* complex were discussed by Kilburn (1972). He noted that "the true position is a very complex one". He considered that the most important factor is the distribution of the forms. Since the ranges of the forms overlap, they cannot, by definition, be treated as subspecies as recommended by Orr. Two alternatives suggested by Kilburn were that the forms could be regarded as ecomorphs, or as full species. Of the former alternative, Kilburn notes that, whilst the forms do have fairly characteristic habitats, there is no obvious correlation between habitat and distribution patterns. Thus, regarding the forms simply as ecomorphs would "generate more problems than it would solve". With regard to the second

alternative, Kilburn concedes that, whilst intermediates do occur, these are relatively rare, and that "to lump all five taxa into one species would attribute to these hybrids an importance which in reality is quite unwarranted.". He concludes that some degree of reproductive isolation may be in progress, and that the most practical solution is to accord the forms full specific rank, at least until further study.

Based on the morphometric and electrophoretic studies outlined in Chapters 1 and 3, I consider that five of the species recognised by Barnard (1959) and Kilburn and Rippey (1982), namely *B. papyracea*, *B. pubescens*, *B. lagenaria*, *B. catarrhacta* and *B. cincta*, should be retained. However, the sixth species, *B. limbosa*, has been reduced to a subspecies of *B. cincta*. In addition, two new species, one of which has previously been confused with *B. papyracea*, are described. The object of this Chapter is to provide descriptions and diagnostic features for these taxa.

MATERIALS AND METHODS

The review of the genus is based on specimens collected by the author for the morphometric (Chapter 1) and electrophoretic (Chapter 3) sections of this thesis, as well as specimens from the following institutions: SAM, BMNH, MHNG, ANSP and MNHN (see abbreviations below).

Abbreviations used in the text are as follows:

ANSP = Academy of Natural Sciences, Philadelphia, USA.

BMNH = Natural History Museum London.

ICZN = International Commission on Zoological Nomenclature.

MHNG = Museum National d'Histoire Naturelle, Geneva.

MNHN = Museum National d'Histoire Naturelle, Paris.

NM = Natal Museum, Pietermaritzburg, South Africa.

SAM = South African Museum, Cape Town, South Africa.

UCT = University of Cape Town ecological survey (deposited at SAM).

YD = specimens collected by the author, or by members of the Zoology Dept. at UCT.

SYSTEMATICS

Genus *Burnupena* Iredale, 1918

Burnupena Iredale, 1918: 28.

Type species: *Buccinum porcatum* Gmelin, 1791: 3494. (= *Burnupena cincta* (Röding, 1798))

Diagnosis: Shell ovate, sometimes slightly fusiform, spire low to moderately high. Whorls more or less depressed below the suture. Parietal callus dentiform. Canal short and wide. Fasciole distinct. Periostracum usually present. Spiral sculpture distinct, with early whorls sometimes cancellate. Radula with rectangular central plate armed with 4 to 9 small, peg-like denticles, laterals quadricuspid, cusps curved, outer cusp large, inner three cusps forming a group (see Fig. 2a; Chapter 2). Egg-capsules leaf-shaped, compressed, with apical flap, cemented to substrata in dome-shaped clusters.

Distribution: Endemic to Southern Africa, ranging from Namibia on the west coast to northern Natal on the east coast.

Remarks: In the synonymies given below, the following two remarks are pertinent to most of the species. (1) Under the genus *Cominella*, Adams & Adams (1853) erected the subgenus *Amphissa* (into which they placed one of the members of *Burnupena*), but they listed the species of *Burnupena*, together with other species, under the genus *Cominella*, without designating a subgenus, so in the synonymies that follow, the subgenus *Cominella* is inferred. (2) Stephenson (1948) referred to all of the species of *Burnupena* as *Cominella*, although he acknowledged that they fall under the section *Burnupena*.

***Burnupena catarrhacta* (Gmelin, 1791)**

(Fig. 1a-b)

Buccinum catarrhacta Gmelin, 1791: 3498 (type figured *in* Chemnitz, 1788, pl. 152 (fig. 1455)).

Buccinum catarracta Dillwyn, 1817: 622.

Buccinum delalandii Kiener, 1834: 15, pl. 5 (fig. 14). Deshayes, 1844: 189. Reeve, 1846, pl. 13 (fig. 106).

Buccinum cataracta Krauss, 1848: 119; 1852: 36.

Buccinum delalandi (Kiener) - Krauss, 1848: 120.

Cominella (*Cominella*) *delalandii* (Kiener) Adams & Adams, 1853: 110.

Cominella delalandi (Kiener) Kobelt, 1878: 232. Tryon, 1881: 203, pl. 80 (fig. 413). Bartsch, 1915: 48. Odhner, 1923: 6.

Cominella cataracta (Krauss) Kobelt, 1878: 232.

Cominella testudinea (Martyn, 1784): Tryon, 1881: 204, *partim*, pl. 80 (fig. 415 = *C. cataracta*, *non* fig. 414).

Cominella delalandii (Kiener) - Sowerby, 1892: 10. Cooke, 1917: 229, fig. 13 (radula). Turton, 1932: 52. Stephenson, 1948: 272.

Burnupena delalandii (Kiener) Iredale, 1918: 34. Tomlin, 1926: 291. Barnard, 1959: 165. Kilburn, 1972: 415. Kensley, 1973: 152, fig. 551. Dance, 1974: 149. Day, 1974: 166, fig. Richards, 1981: 60, pl. 32 (fig. 262).

Burnupena delalandei (Kiener) - Peile, 1938: 97, fig. 31 (radula). Barnard, 1951: 69.

Burnupena delalandi (Kiener) - Orr, 1956: 258, pl. 19 (fig. 10 - shell), pl. 20 (fig. 1 - radula), text-fig. 1f (radula).

Burnupena catarrhacta (Gmelin) Kilburn & Rippey, 1982: 94, pl. 21 (fig. 10). Branch *et al.*, 1994: 162 (fig. 76.3).

Non Purpura cataracta Lamarck, 1822: 245. Kiener, 1834: 130, pl. 36, 37 (figs 85, 85a-e). Deshayes, 1844: 81. Reeve, 1846, pl. 9 (fig. 40). ?Sowerby, 1892: 14.

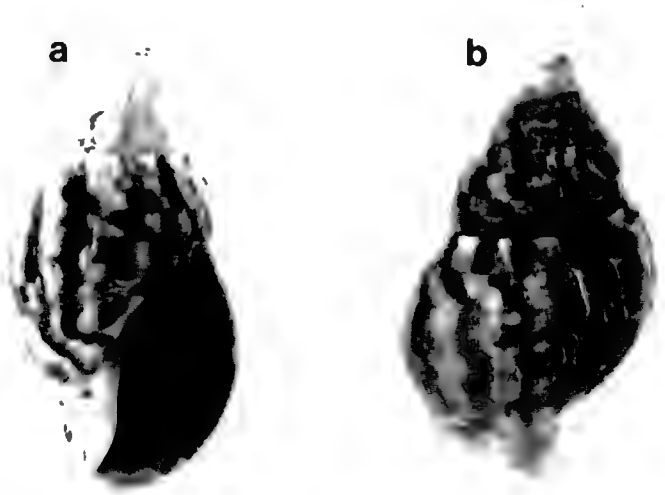

Figure 1. *Burnupena catarrhacta* from Kommetjie. Dimensions given for these and all other figures are shell length and shell width (a) 22.2 x 11.7 mm; (b) 22.9 x 12.3 mm.

Diagnosis: Spire moderately long, angle usually 50° - 60°. Shell width approximately half shell length. Aperture slightly longer than spire. Periphery of body whorl situated approximately half way up the shell. Sculptured by numerous fine spiral striae. Whorls slight to moderately depressed below suture; profile of upper whorls slightly convex. Outer lip thin, usually crenulated at margin, aperture sometimes (but not usually) plicate internally. Dark brown parietal scar usually present. Periostracum thin, yellowish-brown to brown. Shell sometimes with a yellowish-brown, sometimes greenish, ground colour, with well defined dark brown axial flames, often giving a zigzag pattern, other times a spotted appearance - this coloration is most evident in juveniles; shell sometimes uniform dark brown, other times worn. Aperture usually dark brownish-violet, often with a yellowish to whitish outer edge. Differs from all other *Burnupena* species in that the proboscis is coated with a black pigment. Maximum shell length 35 mm.

Distribution: West coast from Hondeklip southwards, and extending round to the south coast as far as Hermanus (Fig. 2). Stephenson's (1948) record from Port Nolloth (north of Hondeklip) requires confirmation as he clearly confused this species with *B. lagenaria* (see remarks below). Tends to congregate in rock crevices, gullies and shallow pools at the mid- to high-tide level. Seldom abundant.

Material examined: Hondeklip (SAM A30969); Lamberts Bay (SAM A36381); Saldanha Bay (ANSP 196197); Langebaan Lagoon (UCT, YD); Oudekraal (SAM A30989, UCT); Kommetjie (SAM A51299, YD); Cape of Good Hope (MNHN - Kiener's syntypes of *Buccinum delalandii*); False Bay (SAM 11117), Simonstown (SAM 4751), Kalk Bay (SAM 2534, SAM 5513), Dalebrook (UCT, YD), Sparks Bay (YD) and Hangklip (SAM A30988) all in False Bay.

Type material: *Buccinum catarrhacta* Gmelin 1791: Gmelin referred to the specimen figured in Chemnitz (1788), but the specimens from the latter collection are lost (D. Kilburn, pers. comm.). *Buccinum delalandii* Kiener, 1834 (MNHN): three syntypes; locality given as Cape of Good Hope.

Remarks: The earliest name given to this species was *Buccinum cataracta* by Chemnitz in 1788, and it was figured in his plate 152 (fig. 1455). However, the complete works of Martini and Chemnitz (1769 to 1795) were placed on the "Official List of Rejected Works in Zoology" by the ICZN, under Opinion 184 of 1958, due to their use of polynomial names for many of the species. Subsequent authors proposed binomial names for Martini and Chemnitz's figures (which have equal status as syntypes - R. Kilburn, pers. comm.) and sometimes used their names (or slight variations thereof), and thus took authorship.

In his synonymy of *Purpura cataracta*, Lamarck (1822) referred to the figures in Chemnitz and to Gmelin's *Buccinum catarrhacta*, but he placed this species in the genus *Purpura*. However, from examination of photographs of his specimens (Fig. 3), kindly made available by the MHNG, it is clear that these are not *Burnupena catarrhacta*. The spire is shorter, and the aperture larger than is typical for *Burnupena catarrhacta*, and they have axial ridges, a feature absent in *B. catarrhacta*. The locality given for Lamarck's specimens was New Zealand. The same applies to Deshayes (1844) since he referred to Lamarck's specimens. Reeve's (1846) figure of *Purpura cataracta* is definitely not *B. catarrhacta*, but resembles *Nucella dubia*. Kiener's (1834) figures of *Purpura cataracta* resemble those of Lamarck's specimens. Sowerby gave no figures, but referred to Reeve's *Purpura cataracta*. Tryon (1881) regarded *Cominella cataracta* (fig. 415), which is the same as the specimen figured by Chemnitz (1788, pl. 152 (fig. 1455) as a colour variation of *Cominella testudinea*, but the latter is a New Zealand species. Thus it is clear that *Purpura cataracta* is unrelated to the South African material, for which the earliest valid name is *Buccinum catarrhacta*.

There has been much confusion in the literature and museum collections between this species and *B. limbosa*, *B.* sp. A and *B. lagenaria* on the West Coast. The *B. delalandii* sample in the reference collection from the UCT ecological survey, identified by Barnard in 1947 (SAM A36381 - locality Lamberts Bay), consists of four specimens. Two of these are *B. lagenaria*, one is *B. catarrhacta*, and the fourth is very small and its identity uncertain. Two samples of *B. delalandii* from Lüderitz (SAM A30521and SAM A30530) comprise specimens of *B.* sp. A, whilst two others from the same locality (SAM A33404 and SAM A3306) are *B. lagenaria*. In a series of papers on the

Figure 3. Specimen identified as *Purpura cataracta* by Lamarck, 1822 (MHNG 1101/65/2).

South African intertidal zones, Bright (1938) noted that *Cominella delalandii* was "almost ubiquitous in damp places" at Port Nolloth, and Stephenson et *al.*, (1940) referred to the "small black whelk *C. delalandii*" as common at Lamberts Bay. Although I have not sampled at either of these two localities, at Groen River, a site between them, *B. lagenaria* was found to be very common, but no *B. catarrhacta* were found. I am of the opinion that the specimens identified as *C. delalandii* in both of these surveys, were in fact *B. lagenaria*.

B. catarrhacta can usually be distinguished from *B. lagenaria* by its more slender shape, longer spire and lack of spiral ridges and by its black pigmented proboscis. When present, the pattern of axial flames is usually a good identifying feature for *B. catarrhacta*. However, certain populations of *B. lagenaria* (especially those on the West Coast) can be confused with *B. catarrhacta*, because they lack spiral ridges and possess an axial pattern. In such cases however, the longer spire of *B. catarrhacta* usually distinguishes this species. Nevertheless, problems in identification do occur at some localities, for example Kommetjie, where the *B. lagenaria* population tends to have relatively longer spires.

Barnard (1959) commented on a "subscalariform" specimen collected in False Bay (SAM 11117) which he identified as *B. lagenaria*. However, the specimen appears to be a normal, although relatively high-spired, specimen of *B. catarrhacta*.

Barnard (1959) commented that "there are no clear-cut conchological differences between this species (*B. catarrhacta*) and *limbosa*", but that "fresh specimens may be separated by coloration". The two species can however, usually be distinguished. Large specimens of *B. cincta limbosa* can be separated by their larger size. Where size cannot be used, *B. catarrhacta* can usually be distinguished by its more acute spire, the dark colour of the aperture, and by the pattern of axial flames. Since there has been much confusion between *B. catarrhacta* and *B. lagenaria*, I suspect that many of the problems experienced by Barnard (1959) in distinguishing between *B. catarrhacta* and *B. limbosa*, were rather a matter of distinguishing between *B. lagenaria* and *B. limbosa*.

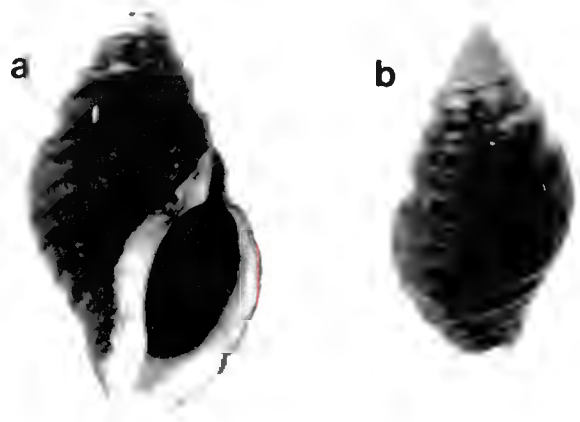

Figure 4a-b. *B. cincta cincta* from Port Elizabeth.
(a) 50.4 x 27.7 mm; (b) 43.3 x 23.1 mm.

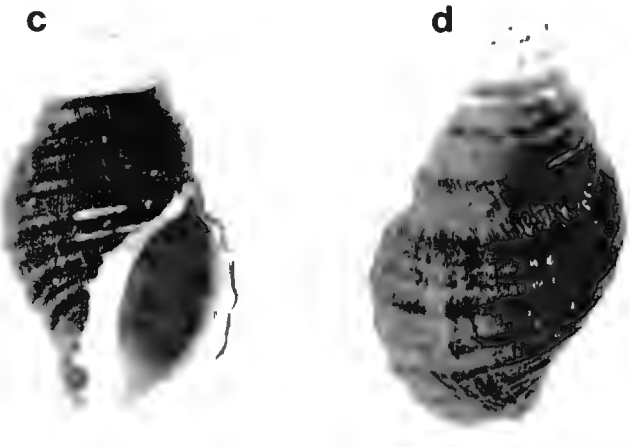

Figure 4c-d. *B. c. cincta* from (c) Dalebrook 44.1 x 23.3 mm;
(d) Plettenberg Bay 44.4 x 26.1 mm (ANSP 196292).

Burnupena (Burnupena) porcata (Gmelin) Thiele, 1929: 315.

Cominella translucida Turton, 1932: 51, pl. 12 (fig. 379).

Cominella cincta (Röding) Turton, 1932: 51. Stephenson, 1948: 272.

Cominella cincta adjacens Turton, 1932: 52, pl. 12 (fig. 381).

? *Burnupena dunkeri* (Küster) Peile, 1938: 98, fig. 33 (radula).

Burnupena papyracea cincta (Röding) Orr, 1956: 254, pl. 19 (figs 5-6 - shell), pl. 20 (fig. 2 - radula), text-fig. 1b-c (radula). Kensley, 1973: 152, fig. 552. Richards, 1981: 60, pl. 32 (fig. 263).

Diagnosis: Spire moderately high, angle usually 60° - 70°. Shell width approximately half shell length. Aperture slightly longer than spire. Periphery of body whorl situated approximately half way up the shell. Sculptured, usually by strong spiral ribs, occasionally weaker, with fine spiral striae on and between ribs. Typically between 4 and 9 spiral ribs on the body whorl. Whorls moderately to strongly depressed below suture; profile of upper whorls stepped to straight. Shell robust. Outer lip undulate at margin (corresponding to ribs), sometimes crenulated at the edge; aperture usually smooth internally, sometimes plicate internally. Pale brown parietal scar usually present. Periostracum thick, fibrous, brown, sometimes stained with green algae. Shell colour usually dull dark brown, occasionally with alternating dark brown and pale whitish-brown dashes on a few of the main spiral ribs; shell sometimes worn or colour completely obscured by the periostracum or by algal growth. Aperture light mauve-brown to pale violet, occasionally white. The proboscis is flesh coloured. Maximum shell length 64 mm.

Distribution: False Bay to Transkei on the South Coast, and sporadically on the West Coast as far as Saldanha Bay (Fig. 5).

Material examined: Saldanha Bay (SAM A4738, ANSP 196198); Sea Point (SAM 2542, SAM A4737); Kommetjie (SAM A51301); Castle Rock (YD), A-Frame (YD), Kalk Bay (SAM 6545), Dalebrook (YD), St. James (SAM A49960), Muizenberg (SAM A51886), Zwartklip (SAM A8605), Gordon's Bay (ANSP 196206), Sparks Bay (YD), Rooiels (YD) and Pringle Bay (YD) all in False Bay; Onrus (ANSP 196205); Hermanus (ANSP 196202, ANSP 196203, ANSP 196204); Cape Agulhas

(ANSP 196296); Still Bay (SAM A30967, SAM A30974); Gouritz River mouth (ANSP 196295); Mossel Bay (SAM 2436, SAM A49959, ANSP 196293, ANSP 196294, YD); Knysna (SAM A4739); Plettenberg Bay (ANSP 196290, ANSP 196292); Jeffrey's Bay (SAM A30966, SAM A49965, ANSP 196297); Port Elizabeth (SAM A4740, YD); East London (SAM A36380); Gonubie (SAM A39271); Qolora (UCT); Port St. Johns (SAM 2507, SAM 11353).

Type material: *Buccinum cinctum* Röding, 1798: type presumed lost (D. Kilburn, pers. comm.); Röding referred to the specimen figured in Chemnitz 1780, but the specimens from the latter collection are lost (D. Kilburn, pers. comm.).
Purpura ligata Lamarck 1822 (MHNG 1152/46 - seen from photographs): three specimens, one of which is a potential lectotype (Fig. 6) as it matches the size and illustration of *Buccinum ligatum* given by Kiener (1834, pl. 15, fig. 15); no locality given.

Remarks: The earliest name given to this species was *Buccinum asperius striatum* by Chemnitz in 1780, and figured in his plate 126 (figs 1213, 1214). However, as noted above (page 198), this name has been invalidated, but the figures were frequently cited and are regarded as the type figures.

Buccinum mexicanum proposed by Bruguière in 1789, is the oldest available name for this species, but it has not been used since, and only listed as a synonym (e.g. Dillwyn, 1817; Reeve, 1846) in some of the older works. An application for this specific name to be placed on the Official Index of Rejected and Invalid Specific Names in Zoology, and that of *cincta* to be placed on the Official List of Specific Names in Zoology, has been submitted to the ICZN, a copy of which is given in Appendix A.

Under Lamarck's *Purpura ligata*, Deshayes (1844) commented that, in agreement with Kiener, this is a *Buccinum*, not a *Purpura*, but he also noted that it is the same as Gmelin's *Buccinum porcatum*.

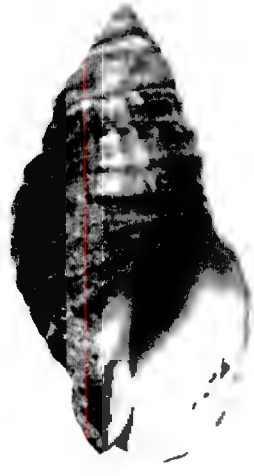

Figure 6. Type specimen of *Buccinum ligatum* Lamarck, 1822. 56.0 x 29.5 mm (MHNG 1152/46 - potential lectotype).

Although I have provisionally placed *Buccinum dunkeri* (and other combinations of Küster's name) as synonymous with this species, its position is uncertain. Tryon (1881) remarked that it is a doubtful species, and even its pertinence to the genus (*Cominella*) is problematical. However, both Orr (1956) and Barnard (1959) synonymized it with *Burnupena cincta*. The radula figured by Peile (1938) is of a juvenile, and he noted that this species can be placed in the genus *Burnupena* based on the lateral teeth, although the central plate (rhachidian) does resemble that of *Afrocominella*.

Thiele (1929) regarded the two genera erected by Iredale (1918), namely *Afrocominella* and *Burnupena*, as sections or subgenera of *Burnupena*, although he should have named *Afrocominella* as the genus as it has line precedence.

Both Orr and Barnard regarded Turton's (1932) *Cominella translucida* as a synonym of *Burnupena tigrina* (= *B. pubescens*). However, the number of spiral ribs on the last whorl (about 7), together with the lack of nodules on the spire (although these could have been worn off as the specimen is clearly worn), make it more likely that this is a specimen of *B. cincta*.

B. cincta cincta is common from the mid-intertidal to the shallow subtidal zone, and is not usually found in exposed positions. In its typical form, there is usually no difficulty in its identification. However, it can be confused with both *B. lagenaria* and *B. cincta limbosa*. Barnard (1959) regarded *B. c. cincta* and *B. lagenaria* as one of the "two most confusing pairs". The two taxa often co-occur, although *B. lagenaria* is generally found higher up the shore, and in more exposed positions. The extreme forms are distinctive, with specimens of *B. c. cincta* having relatively longer spires and strong spiral ribs, whilst those of *B. lagenaria* have short spires and weak ribs. The problem is that phenotypic intermediates are not uncommon (Fig. 7a-b), with specimens of *B. lagenaria* with longer spires and strong ribs apparently more common than specimens of *B. c. cincta* with squat shells and weak ribs (pers. observation). However, they can usually, but not always, be distinguished by the colour of the aperture and of the shell. In *B. c. cincta* the aperture is pale to violet, whilst that of *B. lagenaria* is typically dark. The shell colour of *B. c. cincta* is usually

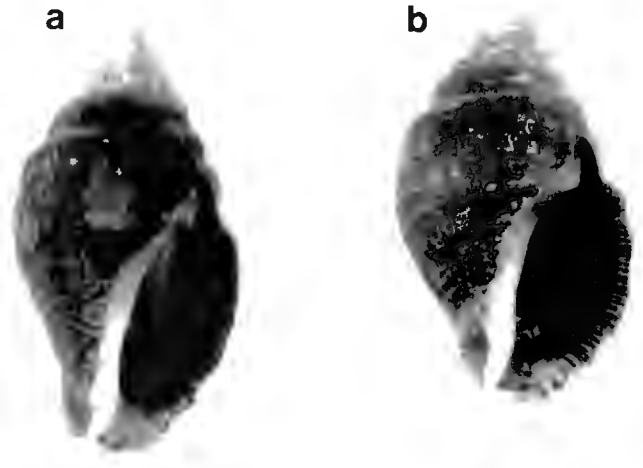

Figure 7. (a) *B.* c. *cincta* and (b) *B. lagenaria* both from Mossel Bay, showing the similarities between the two species. (a) 33.4 x 17.3 mm (b) 31.4 x 17.6 mm.

brown and only occasionally flecked, whilst that of *B. lagenaria* is often flecked or flamed, but sometimes is only brown.

B. c. cincta has been confused with *B. c. limbosa* as they are very similar in most respects, although they differ in the degree of spiral ribs; individuals of the former usually have strong ribs, whilst individuals of *B. c. limbosa* either have smooth shells or a few weak ribs on the lower half of the body whorl. I consider that *limbosa* should be reduced to a subspecies of *B. cincta*, but see remarks below.

***Burnupena cincta limbosa* (Lamarck, 1822)**

(Fig. 8a-b)

Purpura limbosa Lamarck, 1822: 243. *Non* Kiener, 1834: 127, pl. 40 (fig. 95). Deshayes, 1844: 78.

Buccinum limbosum (Lamarck) Reeve, 1846, pl. 5 (fig. 35). Krauss, 1848: 119; 1852: 35. Küster, 1858: 74, pl. 13 (fig. 10).

Cominella limbosa (Lamarck) Gray, 1850: 72. Gray, 1857: 15. Kobelt, 1878: 231. Tryon, 1881: 202, *partim*, pl. 80 (fig. 397, *non* figs 398, 400, 403). Sowerby, 1892: 10. Bartsch, 1915: 48. Cooke, 1917: 229, fig. 15 (radula). Tomlin, 1922: 260. Odhner, 1923: 6. Stephenson, 1948: 273.

Cominella (*Cominella*) *limbosa* (Lamarck) Adams & Adams, 1853: 110, pl. 11 (fig. 6c).

Buccinum (*Cominella*) *limbosa* (Lamarck) Chenu, 1859: 158, fig. 739.

Cominella porcata multilirata Bartsch, 1915: 47, pl. 4 (fig. 6).

Burnupena limbosa (Lamarck) Iredale, 1918: 34. Tomlin, 1926: 291. Peile, 1938: 97; 1939: 270, fig. 38 (radula). Barnard, 1951: 69, pl. 7 (fig. 4); 1959: 164. Kilburn, 1972: 415. Dance, 1974: 149, fig. Day, 1974: 166, fig. Kilburn & Rippey, 1982: 94, pl. 21 (fig. 12).

Cominella cincta multilirata (Bartsch) Turton, 1932: 52.

Cominella lagenaria limbosa (Lamarck) Turton, 1932: 52.

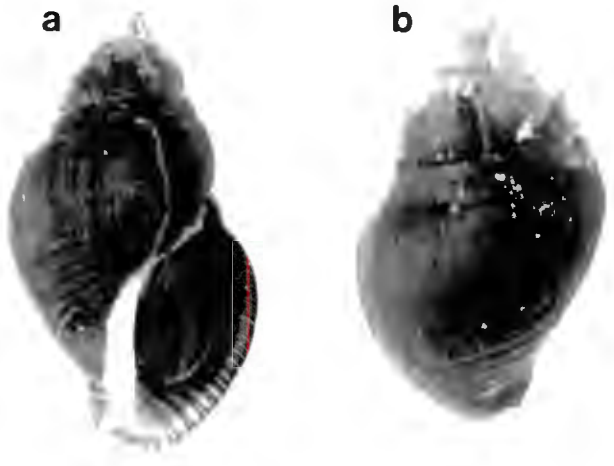

Figure 8. ***B. cincta limbosa*** **from Bakoven. (a) 50.4 x 28.4 mm; (b) 50.0 x 28.5 mm.**

Burnupena papyracea papyracea (*non* Bruguière) Orr, 1956: 252, *partim*, pl. 19 (fig. 1, *non* figs 2-4).

Diagnosis: Spire moderately high, angle usually 60° - 80°. Shell width slightly more than half shell length. Aperture slightly longer than spire. Periphery of body whorl situated approximately half way up the shell. Sculptured usually by numerous fine spiral striae, sometimes with a few weak ribs on the lower half of the body whorl. Whorls slight to moderately depressed below suture; profile of upper whorls slightly convex. Shell robust. Aperture usually plicate internally. Pale brown parietal scar usually present. Periostracum thick, fibrous, brown. Shell colour uniform dark brown, sometimes with thin pale whitish-brown spiral bands, these being most noticeable on the body whorl above the parietal region; shell sometimes worn or colour completely obscured by greenish algal growth. Aperture white to light mauve-brown, sometimes with a darker brownish-violet outer edge. The proboscis is flesh coloured. Maximum shell length 60 mm.

Distribution: West coast from Swakopmond southwards, and extending around Cape Point to Simonstown, with two questionable records at Jeffrey's Bay and Gonubie (Fig. 9).

Material examined: Swakopmond (SAM A29892, YD); Lüderitz (YD); Lamberts Bay (SAM A30986); Paternoster (UCT, YD); Marcus Island (YD); Melkbos (SAM A49957); Blouberg (YD); Table Bay (SAM 5469); Sea Point (UCT, ANSP 196301, BMNH 2258); Oudekraal (UCT, YD); Hout Bay (SAM A4745); Kommetjie (SAM A49956, YD); Cape of Good Hope (BMNH 1840.9.20.26, BMNH 1876.5.19.4); Simonstown (SAM 4748).

Type material: *Purpura limbosa* Lamarck 1822 (MHNG 1101/60 - seen from photographs): two syntypes, one of which is shown in Fig. 10; no locality given.

Remarks: Kiener's (1834) *Purpura limbosa* is clearly not this species, but is a specimen of *B. lagenaria*. Tryon (1881) commented that *Cominella limbosa* might prove to be a well-marked variety of *C. porcata* (i.e. *B. cincta*), an opinion that is substantiated herein.

Figure 10. Type specimen of *Purpura limbosa* Lamarck, 1822. 35.5 x 20.6 mm (MHNG 1101/60/1 - syntype).

B. cincta limbosa inhabits the low intertidal to subtidal zone. It has been reduced to a subspecies of *B. cincta* based on the combined results of morphometric (Chapter 1) and electrophoretic (Chapter 3) studies, together with the fact that *limbosa* and *cincta* appear to occupy different geographic regions. The two taxa are very similar genetically, and differ morphometrically only in the extent of spiral ribbing present: *B. cincta cincta* is typically strongly ribbed, whilst *B. cincta limbosa* is typically smooth shelled. This distinction allows for relatively easy separation.

B. c. *limbosa* has, however, been confused with a number of other species of *Burnupena*. Barnard (1959) regarded this taxon and *B. catarrhacta* as one of the "two most confusing pairs". The distinctions between them have been discussed in the remarks on *B. catarrhacta*. However, as noted above (page 199), Barnard seems to have confused *B. catarrhacta* and *B. lagenaria*. Of a total of 23 specimens from the BMNH which had been identified as *B. limbosa*, 12 are specimens of *B. lagenaria*. The two species can usually be distinguished by (1) the height of the spire, with *B. lagenaria* generally having the shorter spire (2) whether or not the aperture is plicate internally, with *B.* c. *limbosa* almost always plicate, but *B. lagenaria* usually not plicate, and (3) the colour of the shell and the aperture - *B.* c. *limbosa* with a brown shell and pale aperture; *B. lagenaria* with a flecked or brown shell and dark aperture. About one third of the specimens at the SAM identified as *B. limbosa* were not of this species, and of these approximately half are specimens of *B.* sp. A. However, *B.* c. *limbosa* can be usually be distinguished from *B.* sp. A by (1) the height of the spire, which is shorter in *B.* sp. A, (2) the length of the aperture, which is about twice the length of the spire in *B.* sp. A, but only slightly longer than the spire in *B.* c. *limbosa*, (3) the shell colour - dark brown in *B.* c. *limbosa*, but bluish-brown in *B.* sp. A, and (4) in certain populations, the colour of the aperture - always pale in *B.* c. *limbosa*, but some populations of *B.* sp. A have a dark aperture.

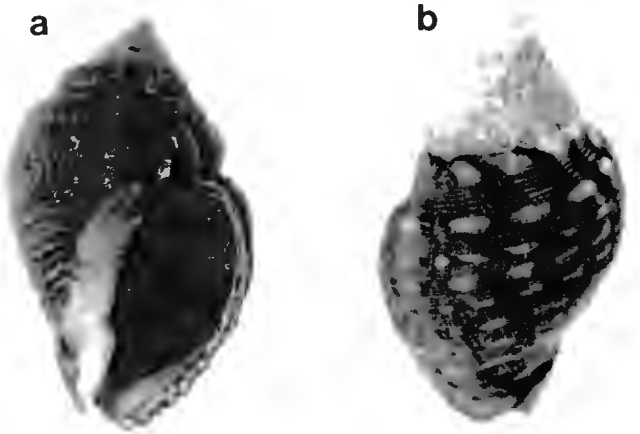

Figure 11a-b. *B. lagenaria* from (a) Port Elizabeth 29.8 x 19.1 mm; (b) Dalebrook 31.8 x 17.9 mm.

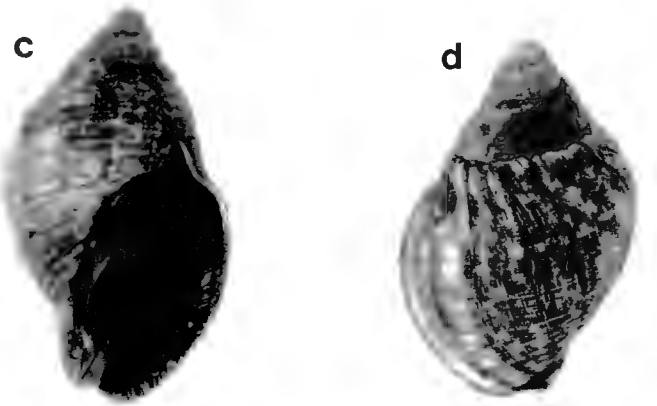

Figure 11c-d. *B. lagenaria* from Groen River (c) 34.3 x 20.2 mm; (d) 32.3 x 19.8 mm.

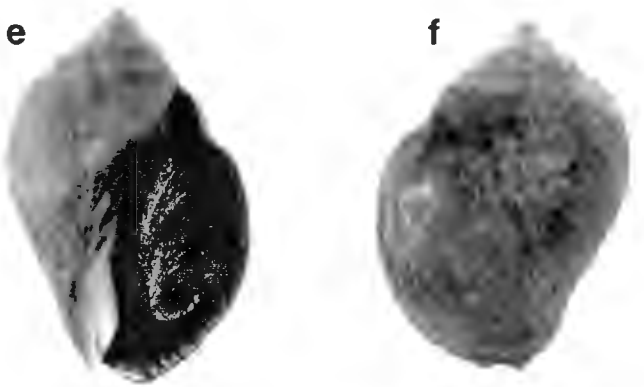

Figure 11e-f. *B. lagenaria* from Durban (e) 23.5 x 15.4 mm; (f) 23.2 x 16.2 mm.

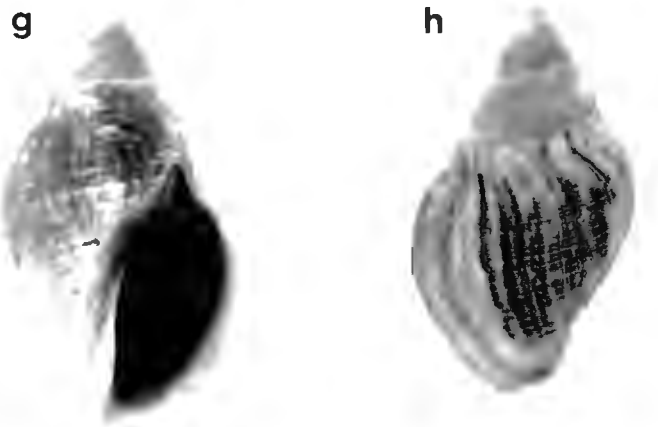

Figure 11g-h. *B. lagenaria* from Kommetjie (g) 28.1 x 15.8 mm; (h) 27.5 x 15.3 mm.

Diagnosis: Shell squat with low spire, angle usually 70° - 80°. Shell width more than half shell length. Aperture approximately twice as long as the spire. Periphery of body whorl situated slightly closer to the apex. Sculpture usually in the form of weak spiral ribs with fine spiral striae on and in between ribs, sometimes with spiral striae and a few weak ribs on the lower half of the body whorl. Typically between 4 and 9 spiral ribs on the body whorl. Whorls moderately to strongly depressed below suture; profile of upper whorls stepped to straight. Outer lip usually crenulated at the edge, aperture smooth internally, sometimes plicate internally. Dark brown parietal scar usually present. Periostracum thick, fibrous, brown, often stained with green algae. Shell colour often with yellowish-brown to brown ground colour, with well defined alternating dark brown and pale whitish-brown to yellowish dashes on the main spiral ribs, sometimes giving a wavy axially flamed appearance; the dashes on the spiral ribs are usually present over most of the body whorl, and are most prominent just below the suture and on the shoulder of the body whorl; sometimes the alternating dashes are not well defined, giving the shell a streaky appearance; shell colour sometimes dark brown, completely lacking the alternating dark and light dashes; upper whorls of shell usually worn. Aperture usually dark brownish-violet, or yellowish-violet, often with a yellowish to whitish outer edge. The proboscis is flesh coloured. Maximum shell length 45 mm.

Distribution: From Lüderitz (in Namibia) on the west coast, extending to northern Natal on the east coast (Fig. 12).

Material examined: Lüderitz (SAM A3306, SAM A33404); Hondeklip (SAM A30969); Groen River (YD); Lamberts Bay (SAM A36381); St. Helena Bay (SAM A49958, UCT); Saldanha Bay (ANSP 196197); Langebaan Lagoon (SAM A30971, UCT, YD); Melkbos (SAM A39292); Blouberg (SAM A38155, YD); Table Bay (SAM 5470, MNHN - syntype of *Buccinum violaceum*); Sea Point (SAM A4737, ANSP 196301, ANSP 196302, BMNH 2258); Mouille Point (SAM A51298); Kommetjie (YD); Platboom (ANSP 196300); Cape of Good Hope (BMNH 1822); Simonstown (ANSP 196200), Kalk Bay (SAM A4741), Dalebrook (YD), Zwartklip (SAM A8606), Gordon's Bay (ANSP 196206), Sparks Bay (YD) and Cape Hangklip (SAM A51302) all in False Bay; Onrus (ANSP 196205);

Hermanus (ANSP 196201, ANSP 196202, YD); Cape Agulhas (ANSP 196296); Breede River estuary (UCT); Still Bay (SAM A30974); Mossel Bay (SAM 2436, ANSP 196293, YD); Plettenberg Bay (ANSP 196290, ANSP 196291, ANSP 196292); Jeffrey's Bay (ANSP 196297, UCT); Port Elizabeth (SAM A36382, ANSP 196289); Richmond (UCT); Kleinmond (UCT); East London (SAM A4742, ANSP 196287, ANSP 196289); Gonubie (SAM A51405); Qolora (SAM A30987, UCT); Coffee Bay (SAM A49961); Mbotyi (SAM A49962); Port Edward (UCT); Port Shepstone (SAM A30061); Durban (YD); Umhalali (SAM A30972).

Type material: *Purpura lagenaria* Lamarck 1822 (MHNG 1101/64 - seen from photographs): three syntypes, one of which is shown in Fig. 13; no locality given.

Remarks: Duclos's (1832) *Purpura lagenaria* is not this species, but resembles *Nucella dubia*. However, he also introduced a new name, *Purpura cucurbita*, the description and figure of which are that of *B. lagenaria*. Kiener (1834) figured what he referred to as varieties of *Purpura lagenaria* (his figs 94a and 94b), but these resemble *Nucella dubia*. His description and figures of *Purpura limbosa* are of *B. lagenaria*. Under *Purpura lagenaria*, Deshayes (1844) commented that this is not *Purpura* but a *Buccinum*, and that Duclos's (1832) *Purpura cucurbita* is the same, but that Duclos's *Purpura lagenaria* is a *Purpura*.

Orr (1956) synonymized *Buccinum violaceum* with *Burnupena cincta*, and Barnard (1959) synonymized it with *B. catarrhacta*. Although the type specimen of *Buccinum violaceum* is small and rather worn (Fig. 14), it appears to be attributable to *B. lagenaria*. Quoy & Gaimard's (1832) illustrations of *Buccinum violaceum* do not resemble the type specimen deposited in the MNHN, but their figures have been repeated in subsequent works (e.g. Kiener, (1834); Tryon, (1881)). Quoy & Gaimard's figures also show a live snail, probably drawn by the ships' artist from a live specimen during the voyage.

B. lagenaria is common to abundant throughout most of its range, and is found in the mid- to high-intertidal zone in pools and exposed habitats. This species not only has the widest

Figure 13. Type specimen of *Purpura lagenaria* Lamarck, 1822. 34.1 x 20.4 mm (MHNG 1101/64/1 - syntype).

Figure 14. Type specimen of *Buccinum violaceum* Quoy & Gaimard, 1832. 25.2 x 16.6 mm (MNHN - syntype).

distribution, but also exhibits greater variation in shell form and coloration (Fig. 11a-h) than the other species in the genus. In terms of shell form, populations on the west and east coasts tend to be squat and have shorter spires and larger apertures, whilst those on the south coast are generally less squat with slightly longer spires and shorter apertures. The population from Kommetjie is an exception, and resembles the south coast populations. The west and east coast populations also tend to have smoother shells, and a higher proportion of shells in which the aperture is plicate internally. In terms of shell colour, the east coast populations are generally dark with no pattern, as are some of the west coast populations. The south coast populations usually exhibit alternating dark and light dashes to a greater or lesser extent. The west coast populations are more variable, with some populations being mostly dark (for example Blouberg), whilst others show varying degrees of axial streaking.

This species has most often been confused with *B. catarrhacta* and *B. cincta*. The features which can be used to distinguish *B. lagenaria* from these two species have already been discussed under the remarks relating to these species.

***Burnupena papyracea* (Bruguière, 1789)**

(Fig. 15a-f)

Buccinum papyraceum Bruguière, 1789: 260 (type figured *in* Chemnitz 1780: pl. 126 (fig. 1212)). Lamarck, 1816: pl. 400 (fig. 3a,b). Dillwyn, 1817: 634. Lamarck, 1822: 264. Kiener, 1834: 8, pl. 4 (fig. 10). Deshayes, 1844: 156. Reeve, 1846, pl. 4 (fig. 24).

Buccinum angelicum Gmelin, 1791: 3494.

Buccinum britanicum Röding, 1798: 113.

Buccinum anglicanum Lamarck, 1822: 264. Kiener, 1834: 7, pl. 4 (fig. 9). Deshayes, 1844: 156. Reeve, 1846, pl. 4 (fig. 23).

Buccinum intinctum Reeve, 1846, pl. 5 (fig. 32). Krauss, 1848: 120; 1852: 36. Küster, 1858: 84, pl. 13 (fig. 12), pl. 15 (figs 3-4).

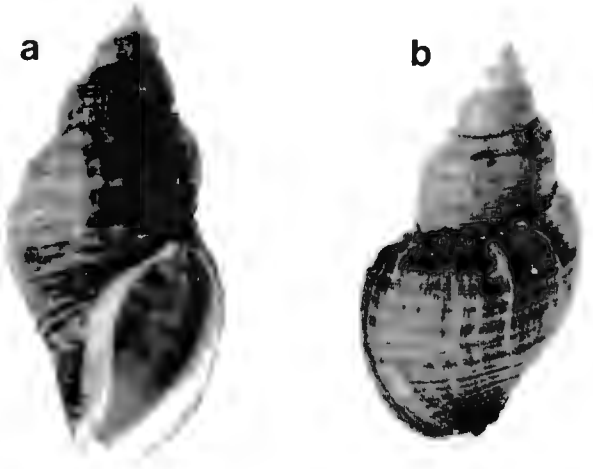

Figure 15a-b. *B. papyracea* from A-Frame (a) 47.6 x 23.4 mm; (b) 44.2 x 22.4 mm.

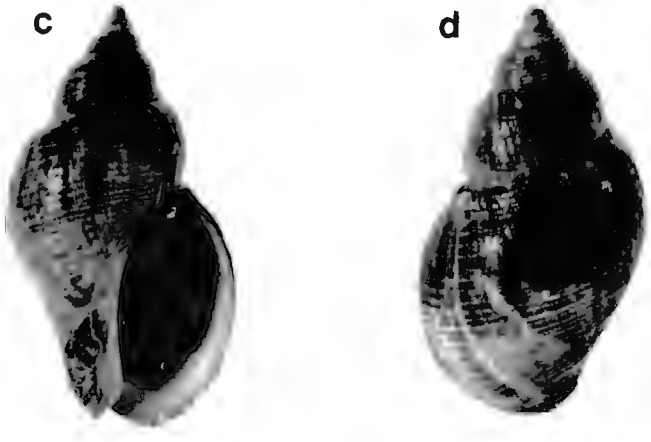

Figure 15c-d. *B. papyracea* from Marcus Island (c) 45.2 x 24.2 mm; (d) 45.7 x 24.5 mm.

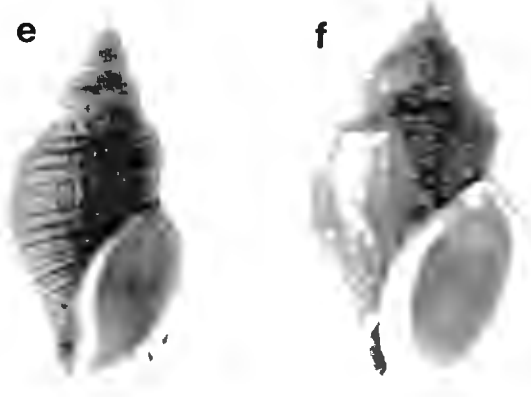

Figure 15e-f. *B. papyracea* from (e) Pringle Bay and (f) Marcus Island, illustrating the differences in ribbing between the False Bay and West Coast individuals. (e) 41.9 x 20.8 mm; (f) 48.5 x 26.3 mm.

Cominella (*Cominella*) *papyracea* (Bruguière) Adams & Adams, 1853: 110.

Cominella (*Amphissa*) *intincta* (Reeve) Adams & Adams, 1853: 111.

Buccinum (*Amphissa*) *intinctum* (Reeve) Chenu, 1859: 158, fig. 738.

Cominella porcata var. *anglicana* (Lamarck) Kobelt, 1878: 231.

Cominella papyracea (Bruguière) Kobelt, 1878: 232. Tryon, 1881: 202, pl. 80 (figs 401, 402 = *C. intincta*). Sowerby, 1892: 10. Bartsch, 1915: 48. Tomlin, 1922: 260. Turton, 1932: 52. Stephenson, 1948: 273.

Cominella porcata (Gmelin) Tryon, 1881: 202, *partim*, pl. 80 (fig. 394 = *C. anglicana*, *non* figs 392, 393, 395, 396, 399, 404).

Cominella anglicana (Lamarck) Bartsch, 1915: 48.

Burnupena papyracea (Bruguière) Peile, 1939: 270. Barnard, 1959: 163. Kilburn, 1972: 415. Day, 1974: 166, fig. Kilburn & Rippey, 1982: 94, pl. 21 (fig. 11). Branch et *al.*, 1994: 162 (fig. 76.5).

Burnupena papyracea papyracea (Bruguière) Orr, 1956: 252, *partim*, pl. 19 (fig. 2, *non* figs 1,3-4 - shell), text-fig. 1d,e (radula). Kensley, 1973: 152, fig. 554. Richards, 1981: 59, pl. 32 (fig. 261).

Diagnosis: Spire moderately high, angle usually 60° - 70°. Shell width approximately half shell length. Aperture slightly longer than spire. Periphery of body whorl situated approximately half way up the shell. Sculptured by either (a) weak to fairly strong spiral ribs, typically between 10 and 14 on the body whorl, with fine spiral striae on and in between ribs, occasionally with a deeper spiral striae on the rib, or (b) by numerous fine spiral striae throughout. Whorls with slight to no depression below suture; upper whorls typically bulging outwards and convex in profile, sometimes with deep sutures. Aperture usually plicate internally, occasionally smooth internally but crenulated at the edge. Pale brown parietal scar usually present, sometimes absent. Periostracum thin, papery, brownish-yellow. Shell colour reddish-brown to brown to pale brown, sometimes mottled, sometimes with pale whitish-brown spiral bands present on part or entire shell, but most noticeable on the body whorl. Aperture usually white, sometimes light mauve-brown. Live specimens are covered by the bryozoan *Alcyonidium nodosum*. The proboscis is flesh coloured. Maximum shell length 58 mm.

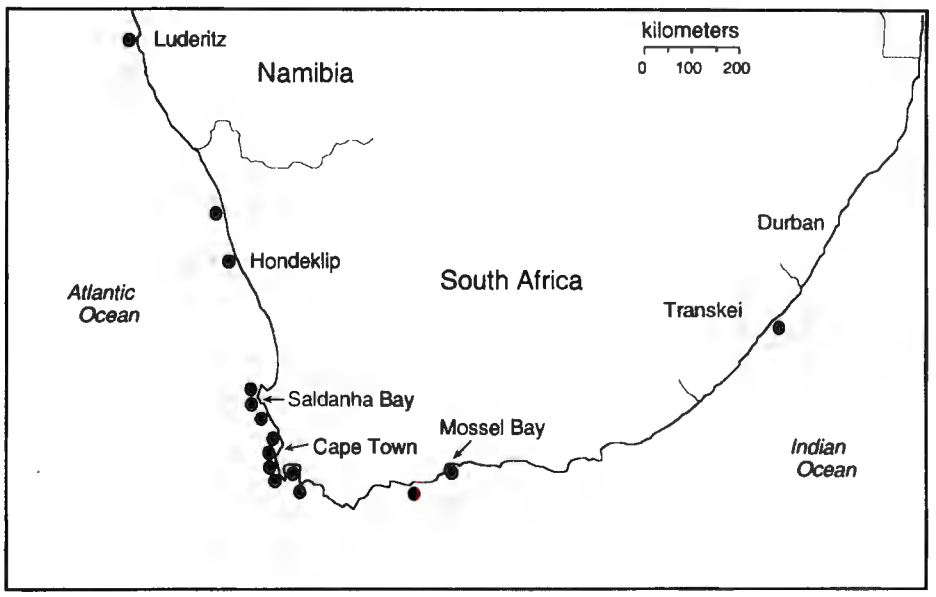

Figure 16. Map showing the distribution of *Burnupena papyracea*. Each black circle represents one or more records.

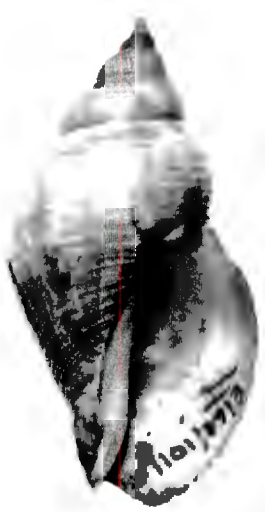

Figure 17. Type specimen of *Buccinum papyraceum* Bruguière, 1789. 49.5 x 25.0 mm (MHNG 1101/89/3 - syntype).

Orr (1956) figured four specimens which she referred to as *B. papyracea papyracea*. However, only her figure 2 is this species. The other figures are as follows: (1) is *B. limbosa*, (3) is *B. lagenaria* and (4) is *B.* sp. A.

Burnupena papyracea is a relatively common species and occurs in deep pools at the low intertidal zone or subtidally in boulder-strewn habitats. Together with *B. pubescens* and *B.* sp. B (see below), it is covered by a bryozoan (which has only been identified with certainty as *Alcyonidium nodosum* in *B. papyracea*), and positive identification of these three species almost always necessitates the removal of the bryozoan. All three species co-occur in False Bay, often in mixed populations. *B. papyracea* can usually be distinguished from both of the other species by the lack of axial ridges on the spire and the convex shape of the whorls (Fig. 18a-f). However, in some specimens of *B. pubescens* and *B.* sp. B the axial ridges are not obvious, making identification more difficult (e.g. Fig. 18g). Specimens of *B. papyracea* never possess alternating dark and pale dashes, which are commonly seen in specimens of *B. pubescens* and *B.* sp. B. *B. papyracea* can also be distinguished from *B.* sp. B by the number of spiral ribs or ridges on the body whorl, with *B. papyracea* typically having between 10 and 14, whereas *B.* sp. B has more that 14.

A lot of four specimens from Muizenberg (SAM A4936) were identified by Barnard (1959) as varieties of *B. pubescens*. He noted that only the early whorls were cancellate, with nodules on the later whorls obsolete. Two of these specimens, are however, attributable to *B. papyracea*, the other two to *B.* sp. B (see below).

***Burnupena pubescens* (Küster, 1858)**

(Fig. 19a-b)

Buccinum tigrinum (*non* Gmelin, 1791) Kiener, 1834: 27, pl. 10 (fig. 32). Krauss, 1848: 120.

Küster, 1858: 80, pl. 14 (fig. 11), pl. 15 (fig. 5).

Cominella (*Cominella*) *tigrina* (Kiener) Adams & Adams, 1853: 110.

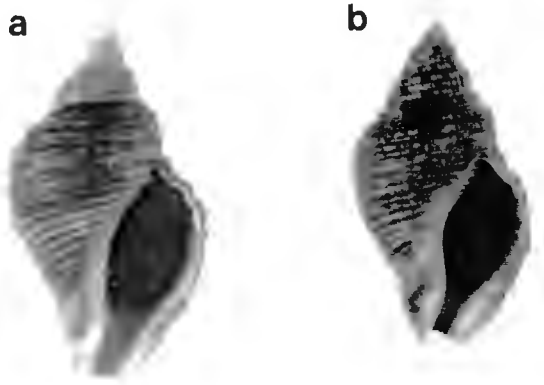

Figure 18a-b. (a) *B. papyracea* and (b) *B. pubescens*, both from A-Frame, illustrating differences between the two species. (a) 27.2 x 15.0 mm; (b) 24.3 x 13.4 mm.

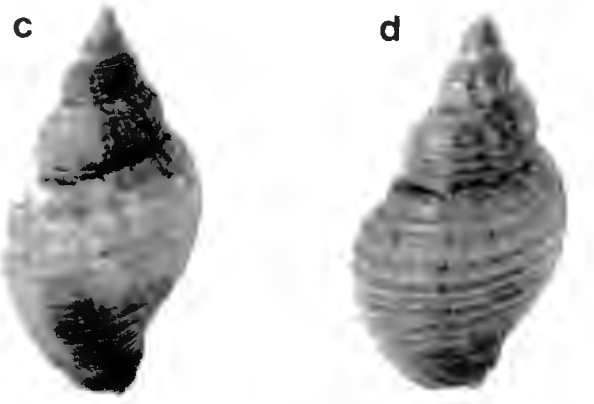

Figure 18c-d. (c) *B. papyracea* and (d) *B. pubescens*, both from A-Frame, showing the differences between the two species. (c) 38.7 x 19.5 mm; (d) 38.1 x 19.8 mm.

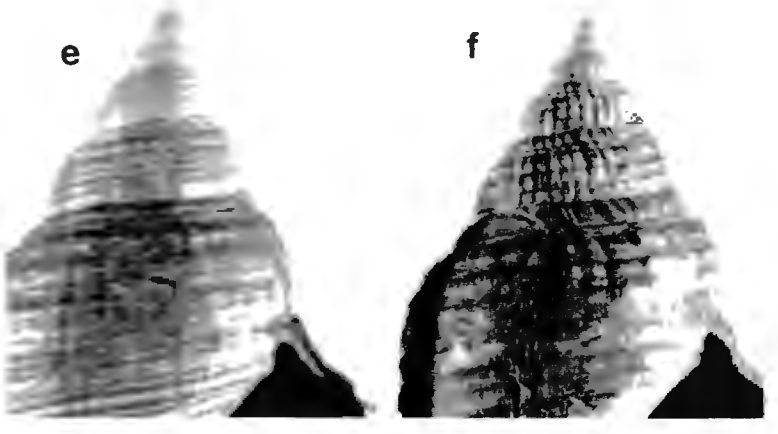

Figure 18e-f. (e) *B. papyracea* and (f) *B. pubescens*, both from A-Frame, showing a close up of the spire to illustrate differences in the sculpture.

Figure 18g. *B. pubescens* from Castle Rock showing a barely cancellate spire. 31.7 x 16.9 mm.

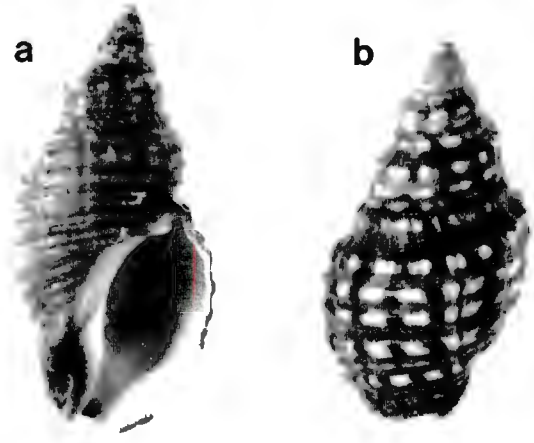

Figure 19. ***B. pubescens*** **from (a) A-Frame 38.1 x 19.8 mm (same specimen as Fig. 18d); (b) East London 36.0 x 18.7 mm (SAM A49966).**

Buccinum pubescens Küster, 1858: 73, pl. 13 (figs 8-9).

Buccinum biseriale Küster, 1858: 80, pl. 14 (fig. 12).

? Buccinum robustum Küster, 1858: 81, pl. 14 (fig. 13).

Cominella porcata var. *pubescens* (Küster) Kobelt, 1878: 231.

Cominella porcata var. *tigrina* (Kiener) Kobelt, 1878: 231. Tryon, 1881: 202, pl. 80 (figs 395 = *C. pubescens*, 396, 399 = *C. robusta*, 404 = *C. biserialis*).

? *Cominella papyracea* var. *robusta* (Küster) Kobelt, 1878: 232.

Cominella biserialis (Küster) Kobelt, 1878: 232. Bartsch, 1915: 47. Turton, 1932: 51.

? *Cominella semisulcata* Sowerby, 1892: 10, pl. 1 (fig. 7)

Cominella tigrina (Kiener) Sowerby, 1892: 11. Bartsch, 1915: 47. *non* Cooke, 1917: 229, fig. 12 (radula). Turton, 1932: 51. Stephenson, 1948: 273.

Afrocominella tigrina (Kiener) Iredale, 1918: 34. Tomlin, 1926: 290.

? *Cominella robusta* (Küster) Turton, 1932: 51.

Burnupena tigrina (Kiener) Peile, 1938: 97, fig. 32 (radula). Barnard, 1959: 166, fig. 32f.

Afrocominella tigrinus (Kiener) - Barnard, 1951: 69.

Burnupena papyracea tigrina (Kiener) Orr, 1956: 255, pl. 19 (figs 7-8 - shell), text-fig. 1a (radula). Kensley, 1973: 152, fig. 555.

Burnupena pubescens (Küster) Kilburn, 1972: 415. Richards, 1981: 60, pl. 32 (fig. 265). Kilburn & Rippey, 1982: 94, pl. 21 (fig. 14). Branch *et al.*, 1994: 162 (fig. 76.6).

Diagnosis: Spire moderately high, angle usually 50° - 60°. Shell width approximately half shell length. Aperture slightly longer than spire. Periphery of body whorl situated approximately half way up the shell. Sculpture usually weak to fairly strong spiral ribs, often nodulate, early whorls cancellate. Typically between 10 and 14 ribs on the body whorl, with fine spiral striae on and between ribs. Whorls slightly to moderately depressed below suture; profile of upper whorls slightly convex. Aperture usually plicate internally. Pale brown parietal scar usually present, sometimes absent. Periostracum thin, papery brownish-yellow. Shell colour yellowish-brown to reddish-brown occasionally pale brown, sometimes with pale whitish-brown spiral bands on the body whorl; raised spiral ribs usually with alternating dark brown and pale whitish-brown dashes,

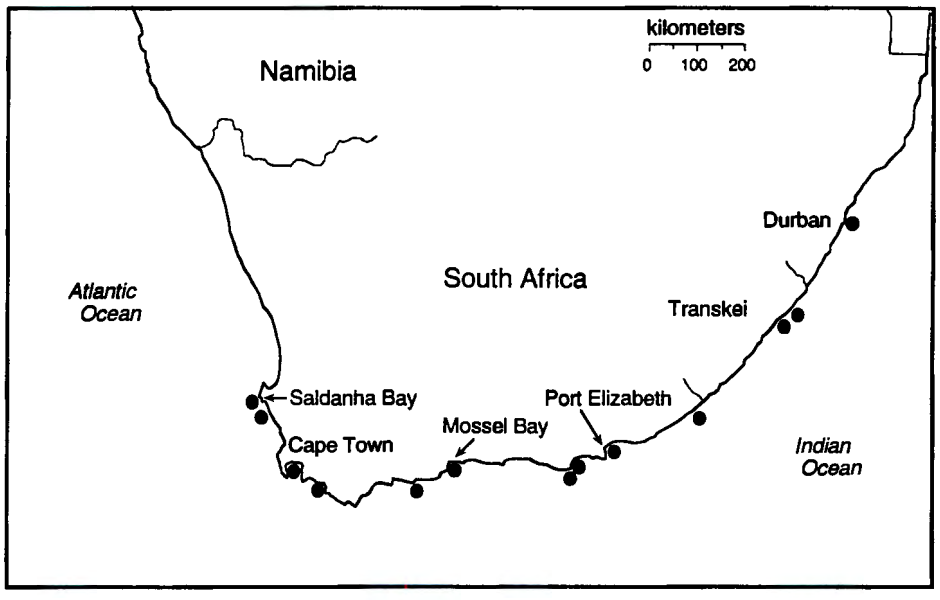

Figure 20. Map showing the distribution of *Burnupena pubescens.* Each black circle represents one or more records.

and differences between it and *B. papyracea* have been discussed above (page 213). *B. pubescens* differs from *B.* sp. B in the number of spiral ribs or ridges on the body whorl - typically 10 to 14 in *B. pubescens* and more than 14 in *B.* sp. B, and in the presence of nodules in *B. pubescens* on the shell other than on the top whorls (Fig. 21a-b). The presence of axial flames on the body whorl is common in *B. pubescens* but has not been observed in *B.* sp. B.

***Burnupena* sp. A sp. nov.**

(Fig. 22a-b)

Burnupena papyracea papyracea (*non* Bruguière) Orr, 1956: 252, *partim*, pl. 19 (fig. 4, *non* figs 1-3).

Burnupena sp. Branch *et al.*, 1994: 162 (fig. 76.4).

Diagnosis: Shell squat with low spire, angle usually 70° - 80°. Shell width more than half shell length. Aperture approximately twice as long as the spire. Periphery of body whorl situated slightly closer to the apex than to the base. Sculptured by numerous fine spiral striae. Whorls with slight to no depression below suture; profile of upper whorls straight. Shell robust. Outer lip thick, aperture usually plicate internally. Dark brown parietal scar usually present. Periostracum thick, fibrous, olive-brown. Shell colour dark bluish-brown, usually with thin pale bluish-grey spiral bands, most visible on the body whorl; shell sometimes worn, upper whorls usually worn. Aperture sometimes pale whitish to mauve-brown, sometimes dark brownish-violet. The proboscis is flesh coloured. Maximum shell length 46 mm.

Description of holotype: Shell sculptured by numerous fine spiral striae. Whorl hardly depressed below the suture. Outer lip crenulated at edge, aperture plicate internally. Dark black brown parietal scar present. Periostracum mostly worn, but at the outer edge of aperture thick, fibrous olive green brown. Shell with alternating dark blue brown and pale blue grey spiral bands. Aperture dark brownish-violet, whitish at anterior tip.

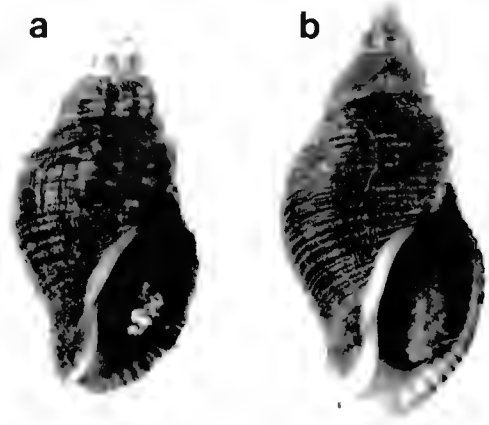

Figure 21. (a) ***B. pubescens*** from Castle Rock 29.6 x 15.2 mm, and (b) ***B.*** sp. B from Miller's Point 33.2 x 16.4 mm (SAM A51965 - paratype).

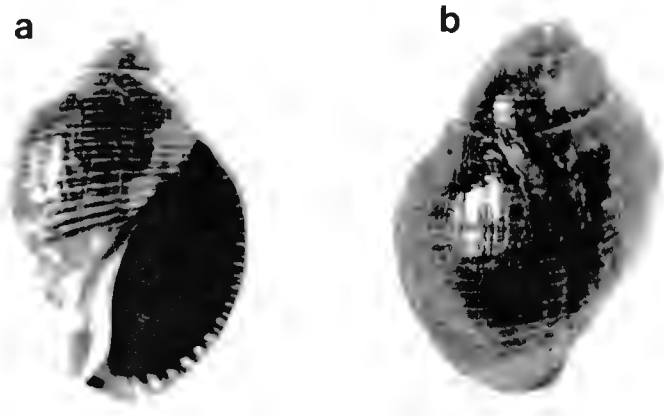

Figure 22. ***Burnupena*** sp. A from Groen River (a) 32.0 x 20.4 mm (SAM A51961 - Holotype); (b) 35.2 x 22.8 mm (SAM A51962 - paratype).

Dimensions: shell length - 32.0mm, shell width - 20.4mm, aperture length - 23.1mm, aperture width - 10.6mm, spire length - 11.6mm.

Distribution: West coast from Lüderitz (in Namibia) southwards, and extending around Cape Point to Simonstown (Fig. 23).

Material examined: Lüderitz (SAM A30521, SAM A30530, YD); Orange River mouth (SAM A29857); Groen River (YD); Lamberts Bay (SAM A30986); Paternoster (ANSP 196303); Marcus Island (YD); Bakoven (YD); Oudekraal (SAM A36383); Cape of Good Hope (BMNH 1840.9.20.26); Simonstown (SAM 4754).

Type material: Holotype, SAM A51961, living, low intertidal zone, rocky shore, Groen River on the west coast of South Africa, collected by G. Branch. **Paratypes 1-15**, SAM A51962, same data; **Paratypes 16-30**, NM V958/T1330, same data.

Remarks: *B.* sp. A inhabits the low intertidal to subtidal zone. In terms of shell shape, it is most similar to *B. lagenaria*, both species being typically squat, with obtuse spires and relatively large apertures. They do differ in the degree of depression below the suture, with *B.* sp. A hardly depressed, and *B. lagenaria* markedly so; internally the aperture of *B.* sp. A is plicate whilst that of *B. lagenaria* is not so; the shell colour differs, with *B.* sp. A dark blue brown with pale spiral bands, and *B. lagenaria* flecked or axially flamed, sometimes brown without flecks, although both species are often worn.

This species has been found in a number of museum collections, but has variously been identified as *B. limbosa* (SAM 4754, SAM A29857, SAM A30986, SAM A36383, BMNH 1840.9.20.26), *B. catarrhacta* (SAM A30521, SAM A30530) or *B. papyracea* (ANSP 196303). However, it is distinct, both morphologically (Chapter 1) and genetically (Chapter 3). Features that distinguish it from *B.* c. *limbosa* have been discussed above (page 206). *B.* sp. A differs from *B. catarrhacta* in a number of respects : it has a totally different shape, being low spired and squat; the

relative size of the aperture is much larger; there is little or no depression below the suture; the aperture is usually plicate internally; the shell is thick and robust; the periostracum is thick and fibrous; the shell is patterned with spiral bands not axial flames. The only case in which *B.* sp. A has been identified as *B. papyracea* was that of Orr (1956 - ANSP 196303), who figured a specimen of this species as one of her examples of *B. papyracea* (Pl. 19, fig. 4). However, she synonymized *B. limbosa* with *B. papyracea*, so it is not clear to which species she initially attributed this specimen. *B.* sp. A can be distinguished from *B. papyracea* by the following characters: (1) the overall shape of the shell of *B.* sp. A is squat with a low spire, whilst that of *B. papyracea* is more slender; (2) the profile of the upper whorls which are convex in *B. papyracea*, but not so in *B.* sp. A; (3) the periostracum in *B. papyracea* is papery, but is fibrous in *B.* sp. A; (4) the shell colour in *B.* sp. A is dark with pale spiral bands, but is reddish- to pale-brown in *B. papyracea*.

***Burnupena* sp. B sp. nov.**

(Fig. 24a-b)

? *Buccinum robustum* Küster, 1858: 81, pl. 14 (fig. 13).

Burnupena tigrina (Kiener) Barnard, 1959: 168, *partim* (reference to two specimens in lot SAM A4936).

Diagnosis: Spire moderately high, angle usually 50° - 60°. Shell width approximately half shell length. Aperture only slightly longer than spire. Periphery of body whorl situated slightly closer to the base of the shell than to the apex. Sculptured by numerous, weak, spiral ridges, often occurring in pairs on the lower half of the body whorl, sometimes with only an inscised groove separating the paired ribs when they lie side by side. Only the early whorls weakly cancellate. Whorls slightly depressed below suture; profile of upper whorls straight. Aperture usually plicate internally. Pale brown parietal scar usually present, sometimes absent. Periostracum thin, fibrous, brownish-yellow. Shell colour orangy-brown to reddish-brown; upper whorls with alternating dark (same colour as the shell) and pale yellowish-brown dashes, giving an axially striped appearance; the body whorl

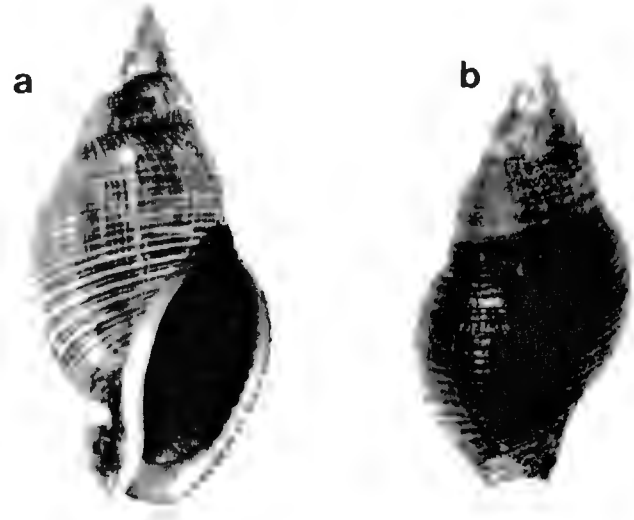

Figure 24. *Burnupena* sp. B from (a) A-Frame 35.5 x 18.2 mm (SAM A51963 - Holotype), and (b) Miller's Point 33.2 x 16.4 mm (SAM A51965 - paratype, same specimen as Fig. 21b).

has no axial stripes but the spiral threads between the ridges are usually paler, especially on the lower half of the body whorl. Aperture white to light mauve-brown. Live specimens are covered by a bryozoan, possibly *Alcyonidium nodosum*. The proboscis is flesh coloured. Maximum shell length 47mm.

Description of holotype: Sculptured by numerous weak spiral ridges which occur in pairs. Top whorls weakly cancellate. Depression below the suture moderate. Outer lip thin, aperture plicate internally. Parietal scar absent. Periostracum thin fibrous yellowish-brown, present only on the lower half of the body whorl. Shell orange brown, with pale brown to whitish spiral bands between the ridges. Upper whorls with alternating pale yellowish-brown and orange brown dashes, with the latter lining up to give a streaky appearance. Aperture pale mauve brown.
Dimensions: shell length - 35.5mm, shell width - 18.2mm, aperture length - 21.2mm, aperture width - 8.6mm, spire length - 17.8mm.

Distribution: False Bay (Fig. 25).

Material examined: Muizenberg (SAM A4936); Castle Rock (YD); Miller's Point (YD); A-Frame (YD); Rooiels (YD).

Type material: Holotype, SAM A51963, living, subtidal 5-10m, boulder strewn, A-Frame, False Bay, collected by YD. **Paratype 1**, SAM A51964, same data; **Paratypes 2-4**, SAM A51965, living, subtidal 5-10m, boulder strewn, Miller's Point, False Bay, collected by YD. **Paratypes 5-6**, NM V957/T1329, same data.

Remarks: As noted above (page 215), the position of Küster's *Buccinum robustum* is unclear. Although I have provisionally placed it with *B. pubescens*, it does bear some resemblance to this undescribed species in having a large number of weak spiral ribs typical of this species. However, although *B.* sp. B does have a weakly cancellate spire, the distinct nodulous cords seen in the figure of *Buccinum robustum* are not present in *B.* sp. B.

As noted above (page 213) a lot of four specimens from Muizenberg (SAM A4936) were identified by Barnard (1959) as varieties of *B. pubescens*, two of which were attributable to *B. papyracea*. The other two specimens were described as having only the early whorls cancellate, and the major costae as scarcely stronger than lirae, both of which are features of this species.

This species occurs subtidally in boulder-strewn habitats, and is very rare. As noted above in the remarks for both *B. papyracea* and *B. pubescens*, the shell is covered by a bryozoan which must be removed from living specimens before they can be identified. This species was originally confused with *B. pubescens* due to the presence of the weakly cancellate spire, but they were found to be genetically distinct. Referral back to the shells of specimens used for electrophoresis revealed that there were also morphological differences between these two species, which have been noted above (page 217). The differences between *B.* sp. B and *B. papyracea* have also been discussed above (page 213).

DISCUSSION

The overall conclusion that was drawn from the results of the morphometric analyses in Chapter 1 was that the degree of intraspecific morphological variation within each of the species was relatively large when compared with the variation within the genus as a whole. Whereas some species can be identified with little difficulty, there will always be some individuals that will be more similar morphometrically to another species; between such species there is a continuum of overlapping forms, with the individuals at either ends being more or less distinctive. The results of the discriminant analyses indicated that about 94% of the individuals examined could be correctly identified, indicating a slightly lower diagnostic value for morphological variation when compared to genetic variation (above 95%), due to the greater susceptibility of the former to environmental conditions.

The results of the examination of the radulae for the species of *Burnupena* presented in Chapter 2 revealed that the radula is of little or no value in distinguishing between the species. Although some differences could be detected, these were not consistent, both within and between species. However, the radula is diagnostic for the genus, as it clearly differs from that of its close relative *Afrocominella*.

The results of the electrophoretic analyses presented in Chapter 3 revealed that, for the most part, the species are genetically well differentiated. Phenograms based on genetic distances indicated that each of the species is a distinct entity. The exception was *B. lagenaria*. The Durban population of this species was, on average, more different from its conspecifics than these were from *B. cincta*. Although clearly genetically distinct, *B. cincta* and *B. lagenaria* did exhibit much lower levels of differentiation relative to the other species. At the other end of the scale, *B. catarrhacta* was very well differentiated from the other species: so much so that it might justifiably be assigned to a separate genus. The genetic distances between the populations of *B. cincta cincta* and *B. c. limbosa* were very low and clearly within the range expected between conspecific

Burnupena papyracea papyracea (*non* Bruguière) Orr, 1956: 252, *partim*, pl. 19 (fig. 1, *non* figs 2-4).

Diagnosis: Spire moderately high, angle usually 60° - 80°. Shell width slightly more than half shell length. Aperture slightly longer than spire. Periphery of body whorl situated approximately half way up the shell. Sculptured usually by numerous fine spiral striae, sometimes with a few weak ribs on the lower half of the body whorl. Whorls slight to moderately depressed below suture; profile of upper whorls slightly convex. Shell robust. Aperture usually plicate internally. Pale brown parietal scar usually present. Periostracum thick, fibrous, brown. Shell colour uniform dark brown, sometimes with thin pale whitish-brown spiral bands, these being most noticeable on the body whorl above the parietal region; shell sometimes worn or colour completely obscured by greenish algal growth. Aperture white to light mauve-brown, sometimes with a darker brownish-violet outer edge. The proboscis is flesh coloured. Maximum shell length 60 mm.

Distribution: West coast from Swakopmond southwards, and extending around Cape Point to Simonstown, with two questionable records at Jeffrey's Bay and Gonubie (Fig. 9).

Material examined: Swakopmond (SAM A29892, YD); Lüderitz (YD); Lamberts Bay (SAM A30986); Paternoster (UCT, YD); Marcus Island (YD); Melkbos (SAM A49957); Blouberg (YD); Table Bay (SAM 5469); Sea Point (UCT, ANSP 196301, BMNH 2258); Oudekraal (UCT, YD); Hout Bay (SAM A4745); Kommetjie (SAM A49956, YD); Cape of Good Hope (BMNH 1840.9.20.26, BMNH 1876.5.19.4); Simonstown (SAM 4748).

Type material: *Purpura limbosa* Lamarck 1822 (MHNG 1101/60 - seen from photographs): two syntypes, one of which is shown in Fig. 10; no locality given.

Remarks: Kiener's (1834) *Purpura limbosa* is clearly not this species, but is a specimen of *B. lagenaria*. Tryon (1881) commented that *Cominella limbosa* might prove to be a well-marked variety of *C. porcata* (i.e. *B. cincta*), an opinion that is substantiated herein.

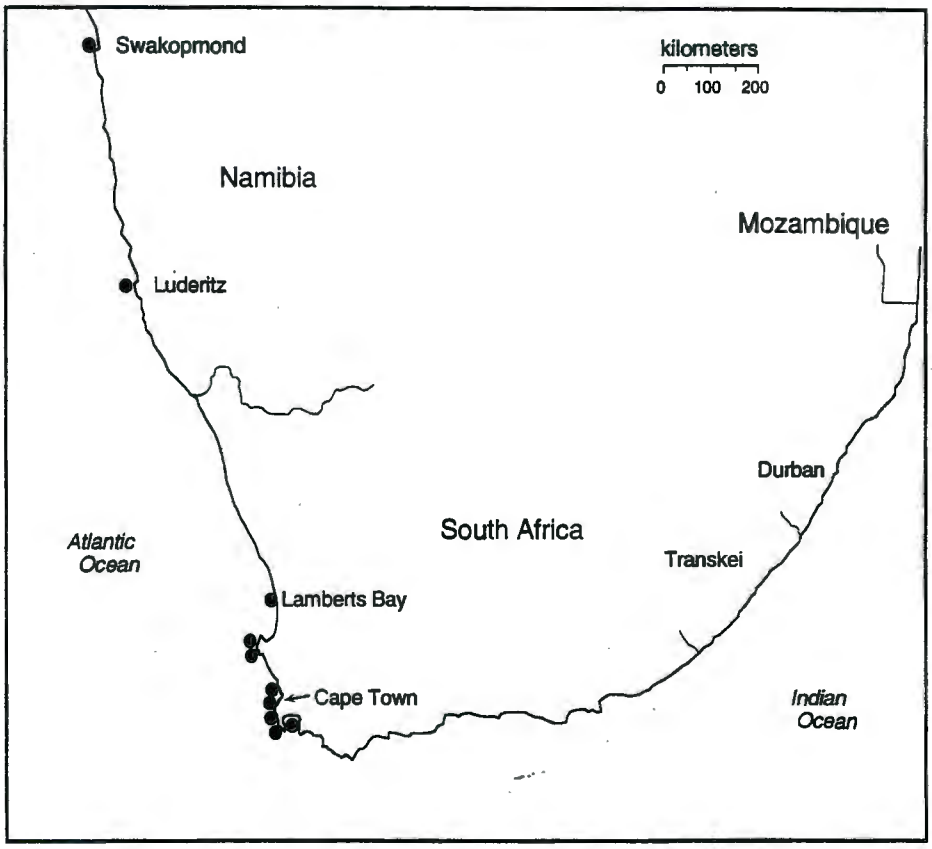

Figure 9. Map showing the distribution of *Burnupena cincta limbosa*. Each black circle represents one or more records.

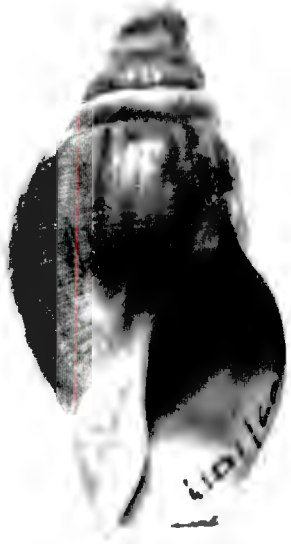

Figure 10. Type specimen of *Purpura limbosa* Lamarck, 1822. 35.5 x 20.6 mm (MHNG 1101/60/1 - syntype).

B. cincta limbosa inhabits the low intertidal to subtidal zone. It has been reduced to a subspecies of *B. cincta* based on the combined results of morphometric (Chapter 1) and electrophoretic (Chapter 3) studies, together with the fact that *limbosa* and *cincta* appear to occupy different geographic regions. The two taxa are very similar genetically, and differ morphometrically only in the extent of spiral ribbing present: *B. cincta cincta* is typically strongly ribbed, whilst *B. cincta limbosa* is typically smooth shelled. This distinction allows for relatively easy separation.

B. c. limbosa has, however, been confused with a number of other species of *Burnupena*. Barnard (1959) regarded this taxon and *B. catarrhacta* as one of the "two most confusing pairs". The distinctions between them have been discussed in the remarks on *B. catarrhacta*. However, as noted above (page 199), Barnard seems to have confused *B. catarrhacta* and *B. lagenaria*. Of a total of 23 specimens from the BMNH which had been identified as *B. limbosa*, 12 are specimens of *B. lagenaria*. The two species can usually be distinguished by (1) the height of the spire, with *B. lagenaria* generally having the shorter spire (2) whether or not the aperture is plicate internally, with *B. c. limbosa* almost always plicate, but *B. lagenaria* usually not plicate, and (3) the colour of the shell and the aperture - *B. c. limbosa* with a brown shell and pale aperture; *B. lagenaria* with a flecked or brown shell and dark aperture. About one third of the specimens at the SAM identified as *B. limbosa* were not of this species, and of these approximately half are specimens of *B.* sp. A. However, *B. c. limbosa* can be usually be distinguished from *B.* sp. A by (1) the height of the spire, which is shorter in *B.* sp. A, (2) the length of the aperture, which is about twice the length of the spire in *B.* sp. A, but only slightly longer than the spire in *B. c. limbosa*, (3) the shell colour - dark brown in *B. c. limbosa*, but bluish-brown in *B.* sp. A, and (4) in certain populations, the colour of the aperture - always pale in *B. c. limbosa*, but some populations of *B.* sp. A have a dark aperture.

***Burnupena lagenaria* (Lamarck, 1822)**

(Fig. 11a-h)

Purpura lagenaria Lamarck, 1822: 245. *Non* Duclos, 1832: 112, pl. 2 (fig. 11). Kiener, 1834: 128, *partim*, pl. 40 (fig. 94, *non* figs 94a, 94b). Deshayes, 1844: 81.

Purpura cucurbita Duclos, 1832: 112, pl. 2 (fig. 12).

Buccinum violaceum Quoy & Gaimard, 1832: 456, pl. 30 (figs 32-34). Kiener, 1834: 33, pl. 8 (fig. 23). Krauss, 1848: 120.

Purpura limbosa (*non* Lamarck) Kiener, 1834: 127, pl. 40 (fig. 95).

Buccinum lagenarium (Lamarck) Reeve, 1846, pl. 5 (figs 33-34). Krauss, 1848: 119. Küster, 1858: 82, pl. 14 (figs 15-16), pl. 15 (figs 1-2).

Cominella lagenaria (Lamarck) Gray, 1850: 72. Gray, 1857: 15. Kobelt, 1878: 231. Sowerby, 1892: 10. Bartsch, 1915: 48. Cooke, 1917: 229, fig. 14 (radula). Tomlin, 1922: 260. Turton, 1932: 52. Stephenson, 1948: 272.

Cominella (*Cominella*) *lagenaria* (Lamarck) Adams & Adams, 1853: 110.

Buccinum (*Cominella*) *lagenaria* (Lamarck) Chenu, 1859: 158, fig. 738.

Cominella violacea (Quoy & Gaimard) Kobelt, 1878: 232. Tryon, 1881: 203, pl. 80 (figs 410-412). Sowerby, 1892: 10.

Cominella limbosa (Lamarck) Tryon, 1881: 202, *partim*, pl. 80 (fig. 398, *non* fig. 397).

Cominella limbosa var. *lagenaria* (Lamarck) Tryon, 1881: 202, pl. 80 (figs 400, 403).

Burnupena lagenaria (Lamarck) Iredale, 1918: 34. Tomlin, 1926: 291. Peile, 1938: 97. Barnard, 1951: 69, pl. 7 (fig. 5); 1959: 162. Kilburn, 1972: 415. Dance, 1974: 149, fig. Day, 1974: 166, fig. Kilburn & Rippey, 1982: 94, pl. 21 (fig. 13), text-fig. 54 (egg capsule). Branch *et al.*, 1994: 162, (fig. 76.2).

Burnupena (*Burnupena*) *lagenaria* (Lamarck) Thiele, 1929: 315, fig. 349 (radula).

Burnupena papyracea papyracea (*non* Bruguière) Orr, 1956: 252, *partim*, pl. 19 (fig. 3, *non* figs 1-2,4 - shell).

Burnupena papyracea lagenaria (Lamarck) Orr, 1956: 256, pl. 19 (fig. 9 - shell), text-fig. 1g,h (radula). Kensley, 1973: 152, fig. 553. Richards, 1981: 60, pl. 32 (fig. 264).

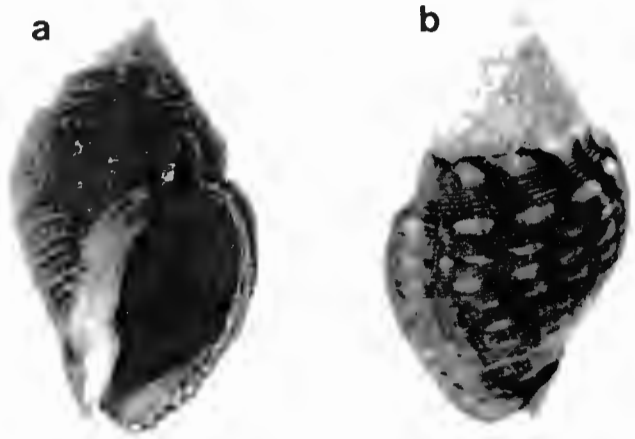

Figure 11a-b. *B. lagenaria* from (a) Port Elizabeth 29.8 x 19.1 mm; (b) Dalebrook 31.8 x 17.9 mm.

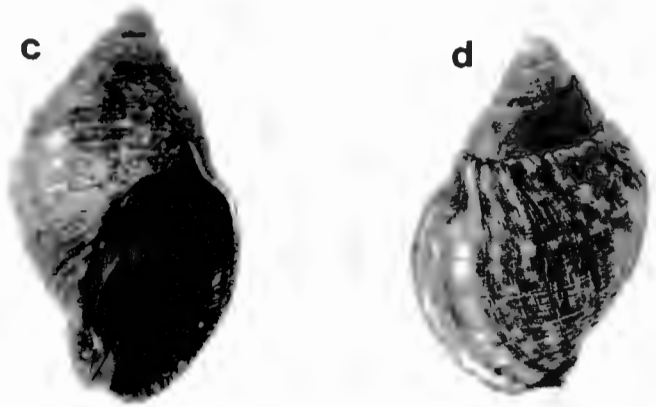

Figure 11c-d. *B. lagenaria* from Groen River (c) 34.3 x 20.2 mm; (d) 32.3 x 19.8 mm.

Figure 11e-f. *B. lagenaria* from Durban (e) 23.5 x 15.4 mm; (f) 23.2 x 16.2 mm.

Figure 11g-h. *B. lagenaria* from Kommetjie (g) 28.1 x 15.8 mm; (h) 27.5 x 15.3 mm.

Diagnosis: Shell squat with low spire, angle usually 70° - 80°. Shell width more than half shell length. Aperture approximately twice as long as the spire. Periphery of body whorl situated slightly closer to the apex. Sculpture usually in the form of weak spiral ribs with fine spiral striae on and in between ribs, sometimes with spiral striae and a few weak ribs on the lower half of the body whorl. Typically between 4 and 9 spiral ribs on the body whorl. Whorls moderately to strongly depressed below suture; profile of upper whorls stepped to straight. Outer lip usually crenulated at the edge, aperture smooth internally, sometimes plicate internally. Dark brown parietal scar usually present. Periostracum thick, fibrous, brown, often stained with green algae. Shell colour often with yellowish-brown to brown ground colour, with well defined alternating dark brown and pale whitish-brown to yellowish dashes on the main spiral ribs, sometimes giving a wavy axially flamed appearance; the dashes on the spiral ribs are usually present over most of the body whorl, and are most prominent just below the suture and on the shoulder of the body whorl; sometimes the alternating dashes are not well defined, giving the shell a streaky appearance; shell colour sometimes dark brown, completely lacking the alternating dark and light dashes; upper whorls of shell usually worn. Aperture usually dark brownish-violet, or yellowish-violet, often with a yellowish to whitish outer edge. The proboscis is flesh coloured. Maximum shell length 45 mm.

Distribution: From Lüderitz (in Namibia) on the west coast, extending to northern Natal on the east coast (Fig. 12).

Material examined: Lüderitz (SAM A3306, SAM A33404); Hondeklip (SAM A30969); Groen River (YD); Lamberts Bay (SAM A36381); St. Helena Bay (SAM A49958, UCT); Saldanha Bay (ANSP 196197); Langebaan Lagoon (SAM A30971, UCT, YD); Melkbos (SAM A39292); Blouberg (SAM A38155, YD); Table Bay (SAM 5470, MNHN - syntype of *Buccinum violaceum*); Sea Point (SAM A4737, ANSP 196301, ANSP 196302, BMNH 2258); Mouille Point (SAM A51298); Kommetjie (YD); Platboom (ANSP 196300); Cape of Good Hope (BMNH 1822); Simonstown (ANSP 196200), Kalk Bay (SAM A4741), Dalebrook (YD), Zwartklip (SAM A8606), Gordon's Bay (ANSP 196206), Sparks Bay (YD) and Cape Hangklip (SAM A51302) all in False Bay; Onrus (ANSP 196205);

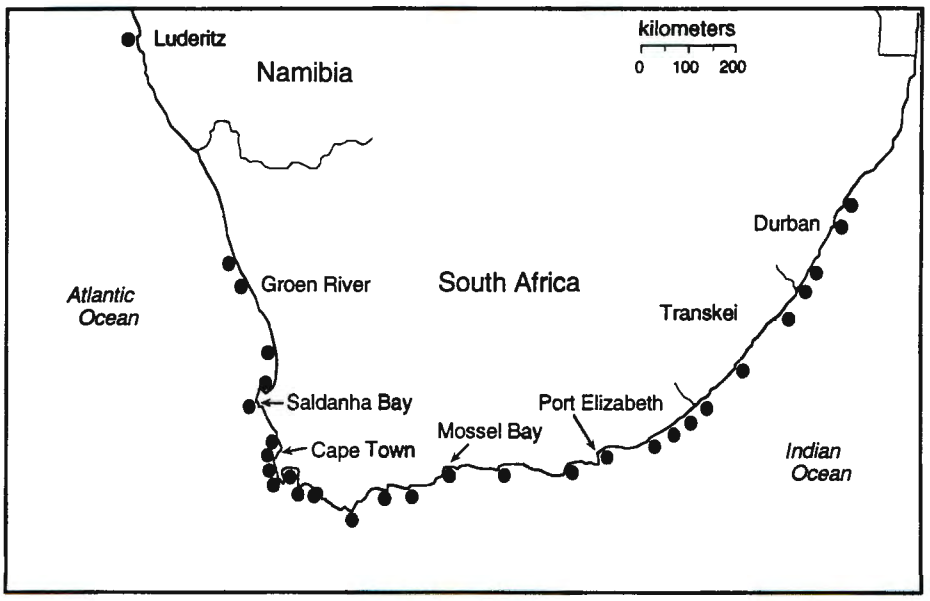

Figure 12. Map showing the distribution of *Burnupena lagenaria.* Each black circle represents one or more records.

Hermanus (ANSP 196201, ANSP 196202, YD); Cape Agulhas (ANSP 196296); Breede River estuary (UCT); Still Bay (SAM A30974); Mossel Bay (SAM 2436, ANSP 196293, YD); Plettenberg Bay (ANSP 196290, ANSP 196291, ANSP 196292); Jeffrey's Bay (ANSP 196297, UCT); Port Elizabeth (SAM A36382, ANSP 196289); Richmond (UCT); Kleinmond (UCT); East London (SAM A4742, ANSP 196287, ANSP 196289); Gonubie (SAM A51405); Qolora (SAM A30987, UCT); Coffee Bay (SAM A49961); Mbotyi (SAM A49962); Port Edward (UCT); Port Shepstone (SAM A30061); Durban (YD); Umhalali (SAM A30972).

Type material: *Purpura lagenaria* Lamarck 1822 (MHNG 1101/64 - seen from photographs): three syntypes, one of which is shown in Fig. 13; no locality given.

Remarks: Duclos's (1832) *Purpura lagenaria* is not this species, but resembles *Nucella dubia*. However, he also introduced a new name, *Purpura cucurbita*, the description and figure of which are that of *B. lagenaria*. Kiener (1834) figured what he referred to as varieties of *Purpura lagenaria* (his figs 94a and 94b), but these resemble *Nucella dubia*. His description and figures of *Purpura limbosa* are of *B. lagenaria*. Under *Purpura lagenaria*, Deshayes (1844) commented that this is not *Purpura* but a *Buccinum*, and that Duclos's (1832) *Purpura cucurbita* is the same, but that Duclos's *Purpura lagenaria* is a *Purpura*.

Orr (1956) synonymized *Buccinum violaceum* with *Burnupena cincta*, and Barnard (1959) synonymized it with *B. catarrhacta*. Although the type specimen of *Buccinum violaceum* is small and rather worn (Fig. 14), it appears to be attributable to *B. lagenaria*. Quoy & Gaimard's (1832) illustrations of *Buccinum violaceum* do not resemble the type specimen deposited in the MNHN, but their figures have been repeated in subsequent works (e.g. Kiener, (1834); Tryon, (1881)). Quoy & Gaimard's figures also show a live snail, probably drawn by the ships' artist from a live specimen during the voyage.

B. lagenaria is common to abundant throughout most of its range, and is found in the mid- to high-intertidal zone in pools and exposed habitats. This species not only has the widest

Figure 13. Type specimen of *Purpura lagenaria* Lamarck, 1822. 34.1 x 20.4 mm (MHNG 1101/64/1 - syntype).

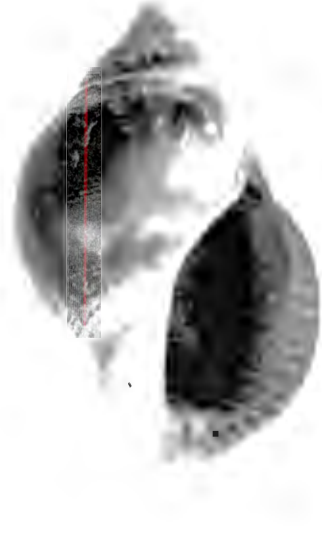

Figure 14. Type specimen of *Buccinum violaceum* Quoy & Gaimard, 1832. 25.2 x 16.6 mm (MNHN - syntype).

distribution, but also exhibits greater variation in shell form and coloration (Fig. 11a-h) than the other species in the genus. In terms of shell form, populations on the west and east coasts tend to be squat and have shorter spires and larger apertures, whilst those on the south coast are generally less squat with slightly longer spires and shorter apertures. The population from Kommetjie is an exception, and resembles the south coast populations. The west and east coast populations also tend to have smoother shells, and a higher proportion of shells in which the aperture is plicate internally. In terms of shell colour, the east coast populations are generally dark with no pattern, as are some of the west coast populations. The south coast populations usually exhibit alternating dark and light dashes to a greater or lesser extent. The west coast populations are more variable, with some populations being mostly dark (for example Blouberg), whilst others show varying degrees of axial streaking.

This species has most often been confused with *B. catarrhacta* and *B. cincta*. The features which can be used to distinguish *B. lagenaria* from these two species have already been discussed under the remarks relating to these species.

***Burnupena papyracea* (Bruguière, 1789)**

(Fig. 15a-f)

Buccinum papyraceum Bruguière, 1789: 260 (type figured *in* Chemnitz 1780: pl. 126 (fig. 1212)). Lamarck, 1816: pl. 400 (fig. 3a,b). Dillwyn, 1817: 634. Lamarck, 1822: 264. Kiener, 1834: 8, pl. 4 (fig. 10). Deshayes, 1844: 156. Reeve, 1846, pl. 4 (fig. 24).

Buccinum angelicum Gmelin, 1791: 3494.

Buccinum britanicum Röding, 1798: 113.

Buccinum anglicanum Lamarck, 1822: 264. Kiener, 1834: 7, pl. 4 (fig. 9). Deshayes, 1844: 156. Reeve, 1846, pl. 4 (fig. 23).

Buccinum intinctum Reeve, 1846, pl. 5 (fig. 32). Krauss, 1848: 120; 1852: 36. Küster, 1858: 84, pl. 13 (fig. 12), pl. 15 (figs 3-4).

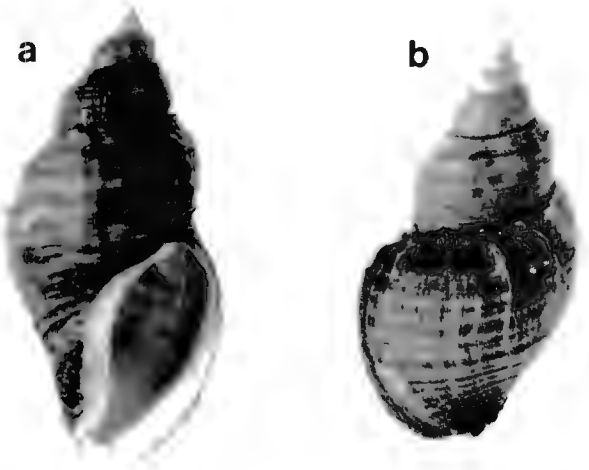

Figure 15a-b. *B. papyracea* from A-Frame (a) 47.6 x 23.4 mm; (b) 44.2 x 22.4 mm.

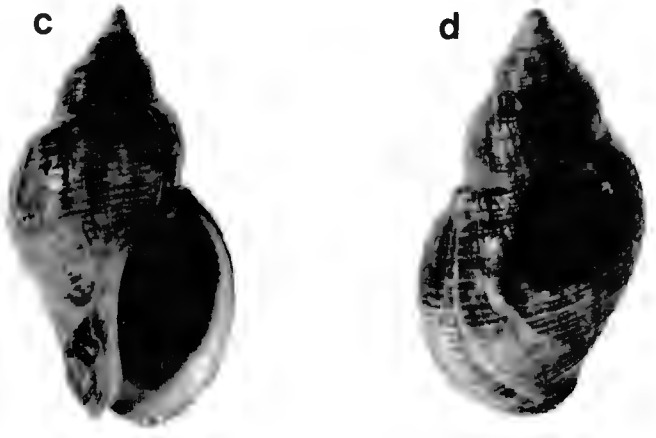

Figure 15c-d. *B. papyracea* from Marcus Island (c) 45.2 x 24.2 mm; (d) 45.7 x 24.5 mm.

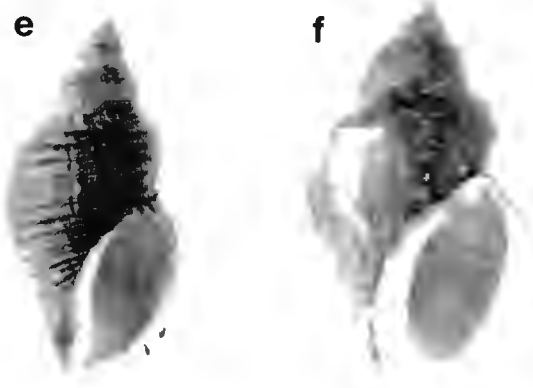

Figure 15e-f. *B. papyracea* from (e) Pringle Bay and (f) Marcus Island, illustrating the differences in ribbing between the False Bay and West Coast individuals. (e) 41.9 x 20.8 mm; (f) 48.5 x 26.3 mm.

Cominella (*Cominella*) *papyracea* (Bruguière) Adams & Adams, 1853: 110.

Cominella (*Amphissa*) *intincta* (Reeve) Adams & Adams, 1853: 111.

Buccinum (*Amphissa*) *intinctum* (Reeve) Chenu, 1859: 158, fig. 738.

Cominella porcata var. *anglicana* (Lamarck) Kobelt, 1878: 231.

Cominella papyracea (Bruguière) Kobelt, 1878: 232. Tryon, 1881: 202, pl. 80 (figs 401, 402 = *C. intincta*). Sowerby, 1892: 10. Bartsch, 1915: 48. Tomlin, 1922: 260. Turton, 1932: 52. Stephenson, 1948: 273.

Cominella porcata (Gmelin) Tryon, 1881: 202, *partim*, pl. 80 (fig. 394 = *C. anglicana*, *non* figs 392, 393, 395, 396, 399, 404).

Cominella anglicana (Lamarck) Bartsch, 1915: 48.

Burnupena papyracea (Bruguière) Peile, 1939: 270. Barnard, 1959: 163. Kilburn, 1972: 415. Day, 1974: 166, fig. Kilburn & Rippey, 1982: 94, pl. 21 (fig. 11). Branch *et al.*, 1994: 162 (fig. 76.5).

Burnupena papyracea papyracea (Bruguière) Orr, 1956: 252, *partim*, pl. 19 (fig. 2, *non* figs 1,3-4 - shell), text-fig. 1d,e (radula). Kensley, 1973: 152, fig. 554. Richards, 1981: 59, pl. 32 (fig. 261).

Diagnosis: Spire moderately high, angle usually 60° - 70°. Shell width approximately half shell length. Aperture slightly longer than spire. Periphery of body whorl situated approximately half way up the shell. Sculptured by either (a) weak to fairly strong spiral ribs, typically between 10 and 14 on the body whorl, with fine spiral striae on and in between ribs, occasionally with a deeper spiral striae on the rib, or (b) by numerous fine spiral striae throughout. Whorls with slight to no depression below suture; upper whorls typically bulging outwards and convex in profile, sometimes with deep sutures. Aperture usually plicate internally, occasionally smooth internally but crenulated at the edge. Pale brown parietal scar usually present, sometimes absent. Periostracum thin, papery, brownish-yellow. Shell colour reddish-brown to brown to pale brown, sometimes mottled, sometimes with pale whitish-brown spiral bands present on part or entire shell, but most noticeable on the body whorl. Aperture usually white, sometimes light mauve-brown. Live specimens are covered by the bryozoan *Alcyonidium nodosum*. The proboscis is flesh coloured. Maximum shell length 58 mm.

Distribution: From Lüderitz (in Namibia) on the west coast, extending to Transkei on the south coast (Fig. 16), although it is not common on the south coast.

Material examined: Lüderitz (SAM 31261, SAM A33405, UCT, YD); Buffels River (UCT); Hondeklip (UCT); Paternoster (ANSP 196303); Marcus Island (YD); Langebaan Lagoon (UCT): Dassen Island (SAM 6879, SAM 6880); Blouberg (SAM A49964, YD); Three Anchor Bay (SAM A4744); Sea Point (SAM 2609, ANSP 196301); Bakoven (YD); Llundudno (YD); Platboom (ANSP 196300); Cape of Good Hope (BMNH 1564); Simonstown (SAM 4749, ANSP 196200), St. James (SAM A36384), Muizenberg (SAM A4936); Castle Rock (YD), A-Frame (YD) and Pringle Bay (YD) all in False Bay; Still Bay (SAM A30981); Mossel Bay (SAM 2435); Port St. Johns (SAM 2504, SAM 2505).

Type material: *Buccinum papyraceum* Bruguière, 1789 (MHNG 1101/89 - seen from photographs): three specimens, with two probable syntypes (see remarks below), one of which (Fig. 17) matches the description given by Bruguière, as well as the figure in Lamarck, 1816 (pl. 400, fig. 3a); locality given as Europe, but certainly incorrect.

Remarks: This species was first described and figured by Chemnitz in 1780, who named it *Buccinum anglicanum elongatum*. As noted above (page 198) regarding the works of Chemnitz, the name is invalid, although a number of subsequent authors used the name *"anglicanum"* or a variation of it. Bruguière (1789) described this species from a shell in Lamarck's collection. The MHNG has a lot of three specimens of this species, which are probably from the Lamarck collection, although only two of them are original syntypes, with the third specimen probably added later on (Y. Finet, pers. comm.). Tryon (1881, fig. 394) regarded *Cominella anglicana* as a synonym of *Cominella porcata*.

This species was neglected by Cooke (1917) and Iredale (1918), and the first mention of it belonging to *Burnupena* came from radula study by Peile, 1939, more than 20 years later.

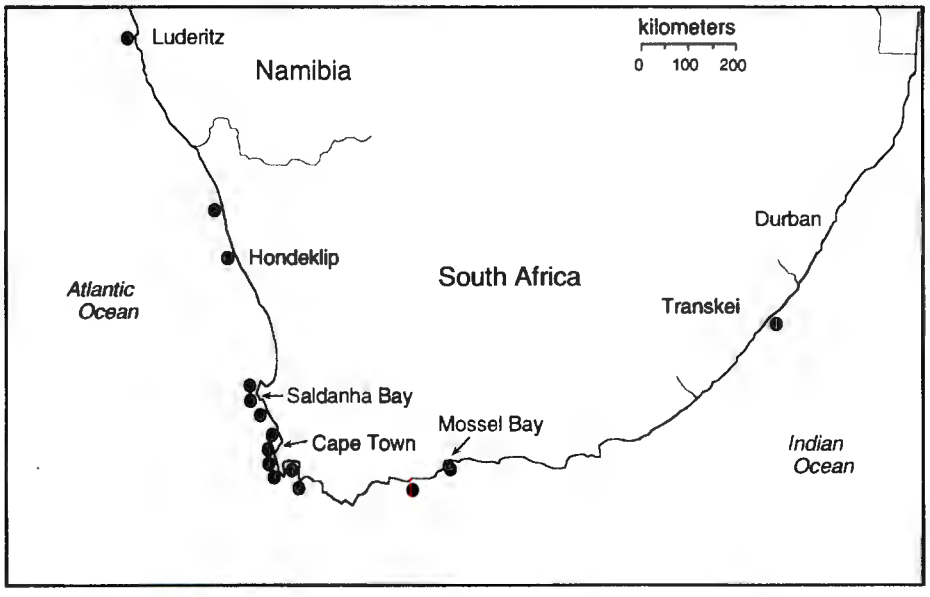

Figure 16. Map showing the distribution of *Burnupena papyracea*. Each black circle represents one or more records.

Figure 17. Type specimen of *Buccinum papyraceum* Bruguière, 1789. 49.5 x 25.0 mm (MHNG 1101/89/3 - syntype).

Orr (1956) figured four specimens which she referred to as *B. papyracea papyracea*. However, only her figure 2 is this species. The other figures are as follows: (1) is *B. limbosa*, (3) is *B. lagenaria* and (4) is *B.* sp. A.

Burnupena papyracea is a relatively common species and occurs in deep pools at the low intertidal zone or subtidally in boulder-strewn habitats. Together with *B. pubescens* and *B.* sp. B (see below), it is covered by a bryozoan (which has only been identified with certainty as *Alcyonidium nodosum* in *B. papyracea*), and positive identification of these three species almost always necessitates the removal of the bryozoan. All three species co-occur in False Bay, often in mixed populations. *B. papyracea* can usually be distinguished from both of the other species by the lack of axial ridges on the spire and the convex shape of the whorls (Fig. 18a-f). However, in some specimens of *B. pubescens* and *B.* sp. B the axial ridges are not obvious, making identification more difficult (e.g. Fig. 18g). Specimens of *B. papyracea* never possess alternating dark and pale dashes, which are commonly seen in specimens of *B. pubescens* and *B.* sp. B. *B. papyracea* can also be distinguished from *B.* sp. B by the number of spiral ribs or ridges on the body whorl, with *B. papyracea* typically having between 10 and 14, whereas *B.* sp. B has more that 14.

A lot of four specimens from Muizenberg (SAM A4936) were identified by Barnard (1959) as varieties of *B. pubescens*. He noted that only the early whorls were cancellate, with nodules on the later whorls obsolete. Two of these specimens, are however, attributable to *B. papyracea*, the other two to *B.* sp. B (see below).

***Burnupena pubescens* (Küster, 1858)**

(Fig. 19a-b)

Buccinum tigrinum (*non* Gmelin, 1791) Kiener, 1834: 27, pl. 10 (fig. 32). Krauss, 1848: 120. Küster, 1858: 80, pl. 14 (fig. 11), pl. 15 (fig. 5).

Cominella (*Cominella*) *tigrina* (Kiener) Adams & Adams, 1853: 110.

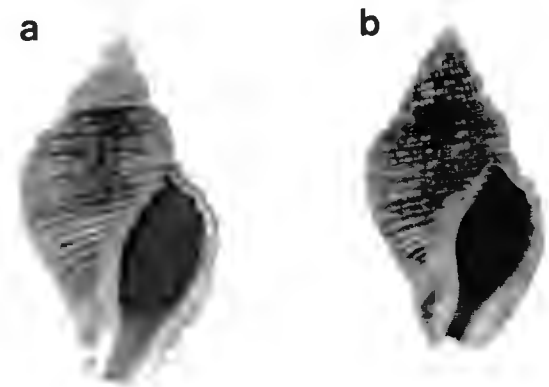

Figure 18a-b. (a) *B. papyracea* and (b) *B. pubescens*, both from A-Frame, illustrating differences between the two species. (a) 27.2 x 15.0 mm; (b) 24.3 x 13.4 mm.

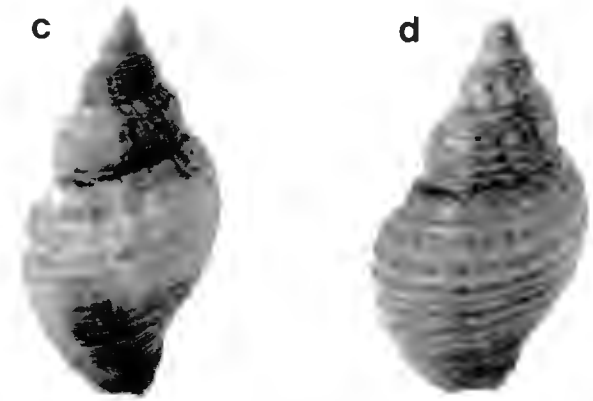

Figure 18c-d. (c) *B. papyracea* and (d) *B. pubescens*, both from A-Frame, showing the differences between the two species. (c) 38.7 x 19.5 mm; (d) 38.1 x 19.8 mm.

Figure 18e-f. (e) *B. papyracea* and (f) *B. pubescens*, both from A-Frame, showing a close up of the spire to illustrate differences in the sculpture.

Figure 18g. *B. pubescens* from Castle Rock showing a barely cancellate spire. 31.7 x 16.9 mm.

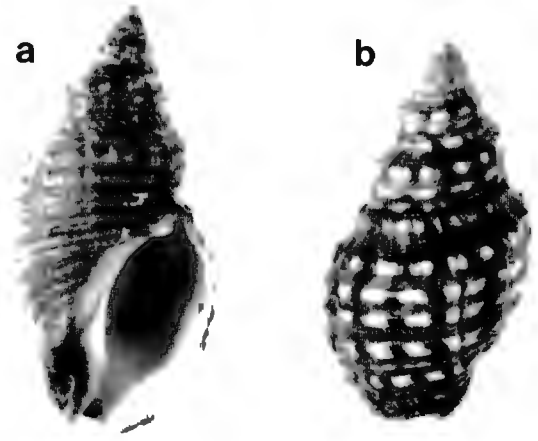

Figure 19. ***B. pubescens*** **from (a) A-Frame 38.1 x 19.8 mm (same specimen as Fig. 18d); (b) East London 36.0 x 18.7 mm (SAM A49966).**

Buccinum pubescens Küster, 1858: 73, pl. 13 (figs 8-9).

Buccinum biseriale Küster, 1858: 80, pl. 14 (fig. 12).

? Buccinum robustum Küster, 1858: 81, pl. 14 (fig. 13).

Cominella porcata var. *pubescens* (Küster) Kobelt, 1878: 231.

Cominella porcata var. *tigrina* (Kiener) Kobelt, 1878: 231. Tryon, 1881: 202, pl. 80 (figs 395 = *C. pubescens*, 396, 399 = *C. robusta*, 404 = *C. biserialis*).

? *Cominella papyracea* var. *robusta* (Küster) Kobelt, 1878: 232.

Cominella biserialis (Küster) Kobelt, 1878: 232. Bartsch, 1915: 47. Turton, 1932: 51.

? Cominella semisulcata Sowerby, 1892: 10, pl. 1 (fig. 7)

Cominella tigrina (Kiener) Sowerby, 1892: 11. Bartsch, 1915: 47. *non* Cooke, 1917: 229, fig. 12 (radula). Turton, 1932: 51. Stephenson, 1948: 273.

Afrocominella tigrina (Kiener) Iredale, 1918: 34. Tomlin, 1926: 290.

? Cominella robusta (Küster) Turton, 1932: 51.

Burnupena tigrina (Kiener) Peile, 1938: 97, fig. 32 (radula). Barnard, 1959: 166, fig. 32f.

Afrocominella tigrinus (Kiener) - Barnard, 1951: 69.

Burnupena papyracea tigrina (Kiener) Orr, 1956: 255, pl. 19 (figs 7-8 - shell), text-fig. 1a (radula). Kensley, 1973: 152, fig. 555.

Burnupena pubescens (Küster) Kilburn, 1972: 415. Richards, 1981: 60, pl. 32 (fig. 265). Kilburn & Rippey, 1982: 94, pl. 21 (fig. 14). Branch *et al.*, 1994: 162 (fig. 76.6).

Diagnosis: Spire moderately high, angle usually 50° - 60°. Shell width approximately half shell length. Aperture slightly longer than spire. Periphery of body whorl situated approximately half way up the shell. Sculpture usually weak to fairly strong spiral ribs, often nodulate, early whorls cancellate. Typically between 10 and 14 ribs on the body whorl, with fine spiral striae on and between ribs. Whorls slightly to moderately depressed below suture; profile of upper whorls slightly convex. Aperture usually plicate internally. Pale brown parietal scar usually present, sometimes absent. Periostracum thin, papery brownish-yellow. Shell colour yellowish-brown to reddish-brown occasionally pale brown, sometimes with pale whitish-brown spiral bands on the body whorl; raised spiral ribs usually with alternating dark brown and pale whitish-brown dashes,

most prominent on the upper half of the body whorl and on the spire, giving a flecked or axially flamed appearance; this pattern is most evident in beach worn specimens. Aperture white to light mauve-brown. Live specimens are covered by a bryozoan, possibly *Alcyonidium nodosum*. The proboscis is flesh coloured. Maximum shell length 41 mm.

Distribution: False Bay to Natal, and sporadically on the West Coast as far as Saldanha Bay (Fig. 20).

Material examined: Saldanha Bay (UCT); Dassen Island (SAM 6886); False Bay (SAM A3532, SAM A4743, SAM A36385), Buffels Bay (ANSP 196299), Castle Rock (YD), A-Frame (YD), Kalk Bay (SAM 4699, SAM 6543), Strandfontein (SAM A49963) and Rooiels (YD) all in False Bay; Hermanus (ANSP 196202); Still Bay (SAM A30978); Mossel Bay (SAM 2435, SAM A4735, SAM A30976, SAM A49967, ANSP 196293); St. Francis Bay (SAM A30980); Jeffrey's Bay (SAM A30977, SAM A49968, SAM A51345); Port Elizabeth (collected for YD by B. Hayes); East London (SAM A4734, SAM A49966); Bonza Bay (SAM A39251); Gonubie (SAM A39252); Port St. Johns (SAM 2532, SAM 11353); Mbotyi (SAM A51403); Durban (SAM 2673).

Type material: *Buccinum pubescens* Küster, 1858: the location of Küster's types is uncertain and they are presumed lost.

Remarks: Krauss (1848) commented that the name *Buccinum tigrinum* had been given to a completely different shell by Gmelin and that this name must be changed. However, it was only changed much later by Kilburn (1972). Küster (1858) erected the name *Buccinum pubescens* for a shell which he regarded as differing from *Buccinum tigrinum* in its proportions and the number of spiral cords.

Küster's (1858) *Buccinum robustum* is problematical: it has been synonymized with *Cominella tigrina* by Tryon (1881) and with *Burnupena papyracea papyracea* by Orr (1956), but Turton (1932) retained it as a separate species. I place it provisionally with *Burnupena pubescens*,

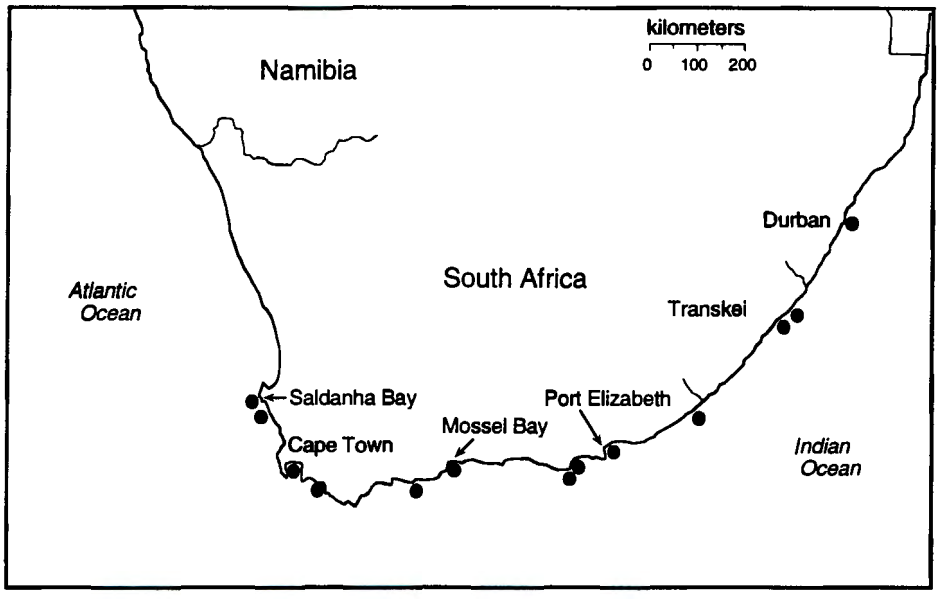

Figure 20. Map showing the distribution of *Burnupena pubescens*. Each black circle represents one or more records.

in that it appears to have nodulous spiral cords, a feature of this species. However, these nodulous cords (Küster, pl. 14 (fig. 13)) are restricted to the area just below the suture, and do not occur elsewhere on the spire. Also *B. robustum* appears to have a large number of weak spiral ribs, more than is usual for this species. Since Küster's types are presumed lost, it is not possible to examine this specimen. Küster recorded the locality as the Cape and Natal coasts, but if this species is not the same as *B. pubescens*, nor *Burnupena* sp. B, to which it also bears some resemblance (see comments below), then it must be regarded as a *species dubium*.

Iredale (1918) assigned *Cominella tigrina* to *Afrocominella* based on the results of a study by Cooke (1917) of the radulae of the genus *Cominella*. However, in a later study, Peile (1938) found that the slide seen by Cooke had been incorrectly labelled, and that the radula figured by Cooke as "*C. tigrina* " was indistinguishable from *Afrocominella elongata*. Peile's study of the radula confirmed that this species is correctly attributable to *Burnupena*.

The identity of Sowerby's (1892) *Cominella semisulcata* is uncertain. Orr (1956) regarded it as a synonym of *B. cincta*, Barnard (1959) placed it with *B. pubescens*, and Kilburn and Rippey (1982) discuss it under *B. cincta*, but comment that its relationship with *B. pubescens* needs investigation. Examination of two lots in the SAM (A3178 & A30980), initially identified as *Cominella semisulcata* but subsequently changed to *C. tigrina*, lead me to conclude that this taxon is most probably a form of *B. pubescens*. All of the specimens in both lots are beach worn. The shells are rather elongate with high spires, are smooth, but shows traces of axial ridges on the top whorls. Barnard commented that Sowerby's figure was not good because the subsutural groove was drawn too deep. A living specimen collected at Port Elizabeth (SAM A51966), does not help, since although it is not worn, the top whorls are obscured by a hard coralline algae. The shell is slender, with an acute spire. The body whorl is brown and the aperture pale.

This species occurs subtidally in boulder-strewn habitats, and is not as common as *B. papyracea*. As noted above, the bryozoan covering needs to be removed from living specimens before they can be identified. *B. pubescens* can be confused with both *B. papyracea* and *B.* sp. B,

and differences between it and *B. papyracea* have been discussed above (page 213). *B. pubescens* differs from *B.* sp. B in the number of spiral ribs or ridges on the body whorl - typically 10 to 14 in *B. pubescens* and more than 14 in *B.* sp. B, and in the presence of nodules in *B. pubescens* on the shell other than on the top whorls (Fig. 21a-b). The presence of axial flames on the body whorl is common in *B. pubescens* but has not been observed in *B.* sp. B.

***Burnupena* sp. A sp. nov.**

(Fig. 22a-b)

Burnupena papyracea papyracea (*non* Bruguière) Orr, 1956: 252, *partim*, pl. 19 (fig. 4, *non* figs 1-3).

Burnupena sp. Branch *et al.*, 1994: 162 (fig. 76.4).

Diagnosis: Shell squat with low spire, angle usually 70° - 80°. Shell width more than half shell length. Aperture approximately twice as long as the spire. Periphery of body whorl situated slightly closer to the apex than to the base. Sculptured by numerous fine spiral striae. Whorls with slight to no depression below suture; profile of upper whorls straight. Shell robust. Outer lip thick, aperture usually plicate internally. Dark brown parietal scar usually present. Periostracum thick, fibrous, olive-brown. Shell colour dark bluish-brown, usually with thin pale bluish-grey spiral bands, most visible on the body whorl; shell sometimes worn, upper whorls usually worn. Aperture sometimes pale whitish to mauve-brown, sometimes dark brownish-violet. The proboscis is flesh coloured. Maximum shell length 46 mm.

Description of holotype: Shell sculptured by numerous fine spiral striae. Whorl hardly depressed below the suture. Outer lip crenulated at edge, aperture plicate internally. Dark black brown parietal scar present. Periostracum mostly worn, but at the outer edge of aperture thick, fibrous olive green brown. Shell with alternating dark blue brown and pale blue grey spiral bands. Aperture dark brownish-violet, whitish at anterior tip.

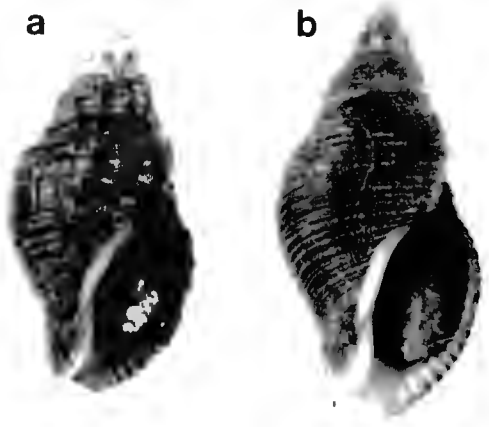

Figure 21. (a) *B. pubescens* from Castle Rock 29.6 x 15.2 mm, and (b) *B.* sp. B from Miller's Point 33.2 x 16.4 mm (SAM A51965 - paratype).

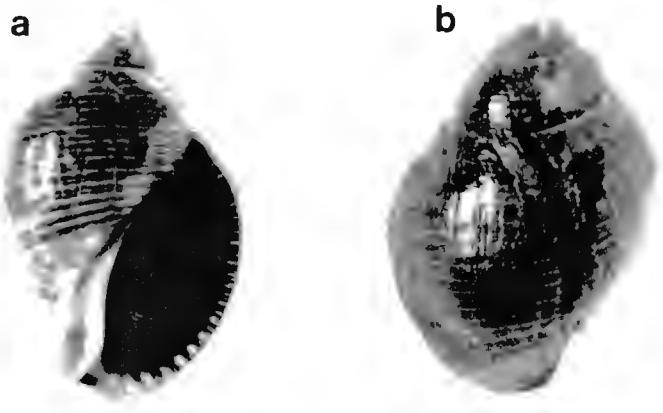

Figure 22. *Burnupena* sp. A from Groen River (a) 32.0 x 20.4 mm (SAM A51961 - Holotype); (b) 35.2 x 22.8 mm (SAM A51962 - paratype).

Dimensions: shell length - 32.0mm, shell width - 20.4mm, aperture length - 23.1mm, aperture width - 10.6mm, spire length - 11.6mm.

Distribution: West coast from Lüderitz (in Namibia) southwards, and extending around Cape Point to Simonstown (Fig. 23).

Material examined: Lüderitz (SAM A30521, SAM A30530, YD); Orange River mouth (SAM A29857); Groen River (YD); Lamberts Bay (SAM A30986); Paternoster (ANSP 196303); Marcus Island (YD); Bakoven (YD); Oudekraal (SAM A36383); Cape of Good Hope (BMNH 1840.9.20.26); Simonstown (SAM 4754).

Type material: Holotype, SAM A51961, living, low intertidal zone, rocky shore, Groen River on the west coast of South Africa, collected by G. Branch. **Paratypes 1-15**, SAM A51962, same data; **Paratypes 16-30**, NM V958/T1330, same data.

Remarks: *B.* sp. A inhabits the low intertidal to subtidal zone. In terms of shell shape, it is most similar to *B. lagenaria*, both species being typically squat, with obtuse spires and relatively large apertures. They do differ in the degree of depression below the suture, with *B.* sp. A hardly depressed, and *B. lagenaria* markedly so; internally the aperture of *B.* sp. A is plicate whilst that of *B. lagenaria* is not so; the shell colour differs, with *B.* sp. A dark blue brown with pale spiral bands, and *B. lagenaria* flecked or axially flamed, sometimes brown without flecks, although both species are often worn.

This species has been found in a number of museum collections, but has variously been identified as *B. limbosa* (SAM 4754, SAM A29857, SAM A30986, SAM A36383, BMNH 1840.9.20.26), *B. catarrhacta* (SAM A30521, SAM A30530) or *B. papyracea* (ANSP 196303). However, it is distinct, both morphologically (Chapter 1) and genetically (Chapter 3). Features that distinguish it from *B. c. limbosa* have been discussed above (page 206). *B.* sp. A differs from *B. catarrhacta* in a number of respects : it has a totally different shape, being low spired and squat; the

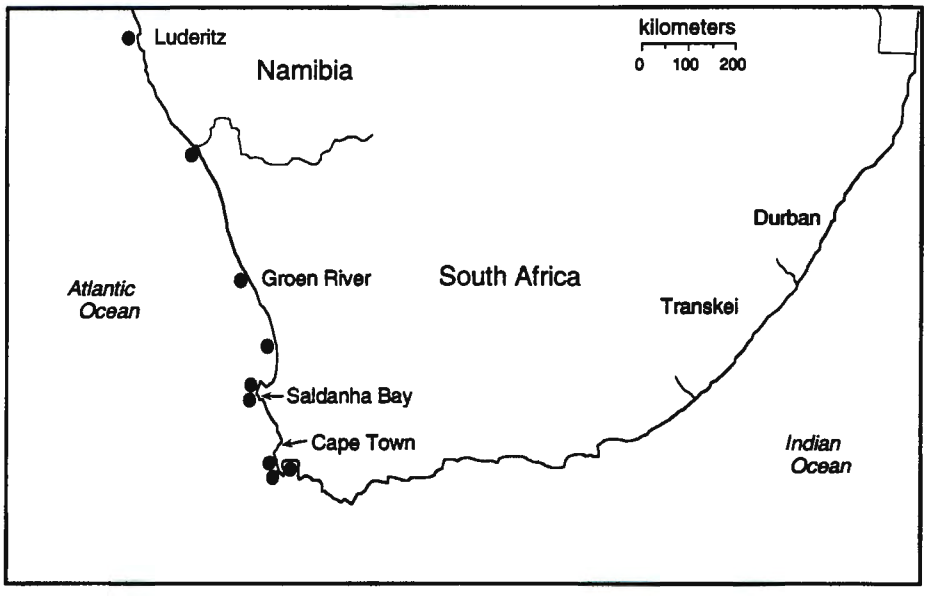

Figure 23. Map showing the distribution of *Burnupena* sp. A. Each black circle represents one or more records.

relative size of the aperture is much larger; there is little or no depression below the suture; the aperture is usually plicate internally; the shell is thick and robust; the periostracum is thick and fibrous; the shell is patterned with spiral bands not axial flames. The only case in which *B.* sp. A has been identified as *B. papyracea* was that of Orr (1956 - ANSP 196303), who figured a specimen of this species as one of her examples of *B. papyracea* (Pl. 19, fig. 4). However, she synonymized *B. limbosa* with *B. papyracea*, so it is not clear to which species she initially attributed this specimen. *B.* sp. A can be distinguished from *B. papyracea* by the following characters: (1) the overall shape of the shell of *B.* sp. A is squat with a low spire, whilst that of *B. papyracea* is more slender; (2) the profile of the upper whorls which are convex in *B. papyracea*, but not so in *B.* sp. A; (3) the periostracum in *B. papyracea* is papery, but is fibrous in *B.* sp. A; (4) the shell colour in *B.* sp. A is dark with pale spiral bands, but is reddish- to pale-brown in *B. papyracea*.

***Burnupena* sp. B sp. nov.**

(Fig. 24a-b)

? *Buccinum robustum* Küster, 1858: 81, pl. 14 (fig. 13).

Burnupena tigrina (Kiener) Barnard, 1959: 168, *partim* (reference to two specimens in lot SAM A4936).

Diagnosis: Spire moderately high, angle usually 50° - 60°. Shell width approximately half shell length. Aperture only slightly longer than spire. Periphery of body whorl situated slightly closer to the base of the shell than to the apex. Sculptured by numerous, weak, spiral ridges, often occurring in pairs on the lower half of the body whorl, sometimes with only an inscised groove separating the paired ribs when they lie side by side. Only the early whorls weakly cancellate. Whorls slightly depressed below suture; profile of upper whorls straight. Aperture usually plicate internally. Pale brown parietal scar usually present, sometimes absent. Periostracum thin, fibrous, brownish-yellow. Shell colour orangy-brown to reddish-brown; upper whorls with alternating dark (same colour as the shell) and pale yellowish-brown dashes, giving an axially striped appearance; the body whorl

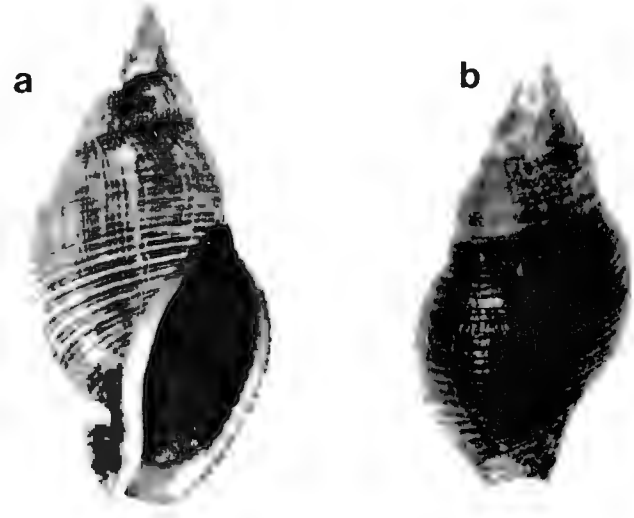

Figure 24. ***Burnupena*** **sp. B from (a) A-Frame 35.5 x 18.2 mm (SAM A51963 - Holotype), and (b) Miller's Point 33.2 x 16.4 mm (SAM A51965 - paratype, same specimen as Fig. 21b).**

has no axial stripes but the spiral threads between the ridges are usually paler, especially on the lower half of the body whorl. Aperture white to light mauve-brown. Live specimens are covered by a bryozoan, possibly *Alcyonidium nodosum*. The proboscis is flesh coloured. Maximum shell length 47mm.

Description of holotype: Sculptured by numerous weak spiral ridges which occur in pairs. Top whorls weakly cancellate. Depression below the suture moderate. Outer lip thin, aperture plicate internally. Parietal scar absent. Periostracum thin fibrous yellowish-brown, present only on the lower half of the body whorl. Shell orange brown, with pale brown to whitish spiral bands between the ridges. Upper whorls with alternating pale yellowish-brown and orange brown dashes, with the latter lining up to give a streaky appearance. Aperture pale mauve brown.
Dimensions: shell length - 35.5mm, shell width - 18.2mm, aperture length - 21.2mm, aperture width - 8.6mm, spire length - 17.8mm.

Distribution: False Bay (Fig. 25).

Material examined: Muizenberg (SAM A4936); Castle Rock (YD); Miller's Point (YD); A-Frame (YD); Rooiels (YD).

Type material: Holotype, SAM A51963, living, subtidal 5-10m, boulder strewn, A-Frame, False Bay, collected by YD. **Paratype 1**, SAM A51964, same data; **Paratypes 2-4**, SAM A51965, living, subtidal 5-10m, boulder strewn, Miller's Point, False Bay, collected by YD. **Paratypes 5-6**, NM V957/T1329, same data.

Remarks: As noted above (page 215), the position of Küster's *Buccinum robustum* is unclear. Although I have provisionally placed it with *B. pubescens*, it does bear some resemblance to this undescribed species in having a large number of weak spiral ribs typical of this species. However, although *B.* sp. B does have a weakly cancellate spire, the distinct nodulous cords seen in the figure of *Buccinum robustum* are not present in *B.* sp. B.

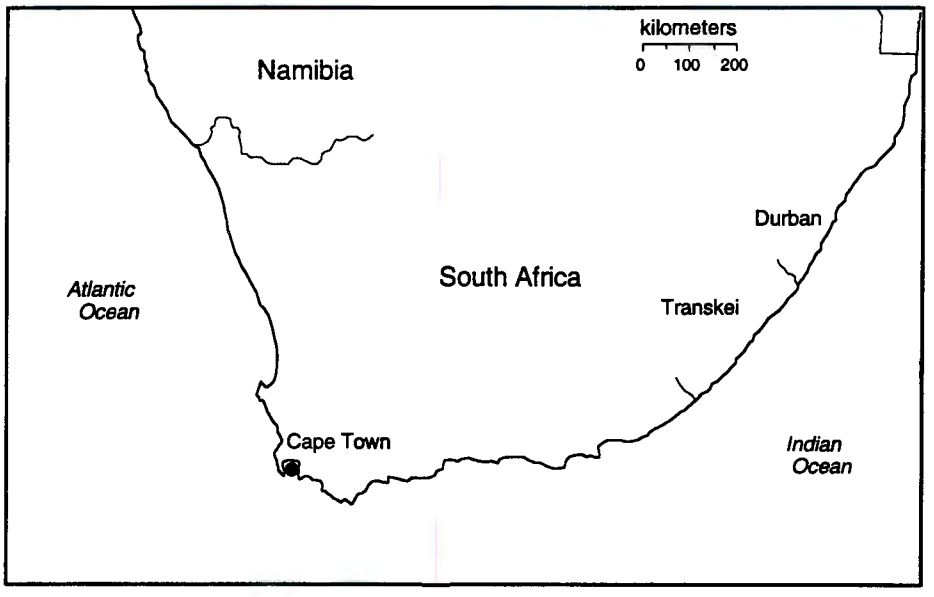

Figure 25. Map showing the distribution of *Burnupena* sp. B. The black circle represents more than one record.

As noted above (page 213) a lot of four specimens from Muizenberg (SAM A4936) were identified by Barnard (1959) as varieties of *B. pubescens*, two of which were attributable to *B. papyracea*. The other two specimens were described as having only the early whorls cancellate, and the major costae as scarcely stronger than lirae, both of which are features of this species.

This species occurs subtidally in boulder-strewn habitats, and is very rare. As noted above in the remarks for both *B. papyracea* and *B. pubescens*, the shell is covered by a bryozoan which must be removed from living specimens before they can be identified. This species was originally confused with *B. pubescens* due to the presence of the weakly cancellate spire, but they were found to be genetically distinct. Referral back to the shells of specimens used for electrophoresis revealed that there were also morphological differences between these two species, which have been noted above (page 217). The differences between *B.* sp. B and *B. papyracea* have also been discussed above (page 213).

Key to the species of the genus *Burnupena*

1a) Early whorls cancellate-nodulose (see Fig. 18e-f); live-collected specimens always covered with a bryozoan 2

b) Early whorls with spiral sculpture only (see Fig. 18e-f); live-collected specimens not covered with a bryozoan except in the case of *B. papyracea* 3

2a) Shell with 10 to 14 spiral ribs on body whorl, often with nodules on shell other than on the spire; shell often with flecked pattern (see Fig. 19b) ..... *B. pubescens*

b) Shell with numerous (usually > 14) weak spiral ridges on the body whorl, never enlarged to ribs, with no nodules, and spire only weakly cancellate; shell with axial flames on early whorls only (see Fig. 24a-b) ..... *B.* sp. B

3a) Shell squat with obtuse spire; aperture length about twice spire length 4

b) Shell with aperture length approximately equal to spire length 5

4a) Depression below the suture moderate to strong (see Fig. 11a,e); aperture usually smooth internally ..... *B. lagenaria*

b) Slight to no depression below the suture (see Fig. 22a); aperture plicate internally ..... *B.* sp. A

5a) Profile of upper whorls distinctly convex (see Figs 15(b,f), 17); periostracum papery; colour relatively uniform, never flamed; live-collected specimens covered with a bryozoan ..... *B. papyracea*

b) Profile of upper whorls only slightly or not convex; periostracum not papery; shell sometimes flamed; live specimens never with bryozoan 6

6a) Shell sculptured by 4 to 9 spiral ribs (see Fig. 4) ..... *B. cincta cincta*

b) Shell with numerous spiral striae, never ribs 7

7a) Aperture pale, plicate internally; shell uniformly brown or tinted green by algal growth; periostracum thick, fibrous; proboscis flesh coloured ..... *B. cincta limbosa*

b) Aperture dark, usually smooth internally; shell often with axial flames; periostracum thin; proboscis pigmented black ..... *B. catarrhacta*

DISCUSSION

The overall conclusion that was drawn from the results of the morphometric analyses in Chapter 1 was that the degree of intraspecific morphological variation within each of the species was relatively large when compared with the variation within the genus as a whole. Whereas some species can be identified with little difficulty, there will always be some individuals that will be more similar morphometrically to another species; between such species there is a continuum of overlapping forms, with the individuals at either ends being more or less distinctive. The results of the discriminant analyses indicated that about 94% of the individuals examined could be correctly identified, indicating a slightly lower diagnostic value for morphological variation when compared to genetic variation (above 95%), due to the greater susceptibility of the former to environmental conditions.

The results of the examination of the radulae for the species of *Burnupena* presented in Chapter 2 revealed that the radula is of little or no value in distinguishing between the species. Although some differences could be detected, these were not consistent, both within and between species. However, the radula is diagnostic for the genus, as it clearly differs from that of its close relative *Afrocominella*.

The results of the electrophoretic analyses presented in Chapter 3 revealed that, for the most part, the species are genetically well differentiated. Phenograms based on genetic distances indicated that each of the species is a distinct entity. The exception was *B. lagenaria*. The Durban population of this species was, on average, more different from its conspecifics than these were from *B. cincta*. Although clearly genetically distinct, *B. cincta* and *B. lagenaria* did exhibit much lower levels of differentiation relative to the other species. At the other end of the scale, *B. catarrhacta* was very well differentiated from the other species: so much so that it might justifiably be assigned to a separate genus. The genetic distances between the populations of *B. cincta cincta* and *B.* c. *limbosa* were very low and clearly within the range expected between conspecific

Table 1. Summary of the features of the species of *Burnupena*. The shell length indicates the size range of the individuals examined in the morphometric analysis.

	species							
Features	*B. catarrhacta*	*B. c. cincta*	*B. c. limbosa*	*B. lagenaria*	*B. papyracea*	*B. pubescens*	*B.* sp. A	*B.* sp. B
spire height	moderate to high	moderate	moderate	low	moderate	moderate to high	low	moderate to high
shell width	approx. half shell length	approx. half shell length	slightly more than half shell length	more than half shell length	approx. half shell length	approx. half shell length	more than half shell length	approx. half shell length
aperture length	slightly longer than spire	slightly longer than spire	slightly longer than spire	approx. twice spire length	slightly longer than spire	slightly longer than spire	approx. twice spire length	slightly longer than spire
periphery of body whorl	approx. midway between base and apex	approx. midway between base and apex	approx. midway between base and apex	closer to apex	approx. midway between base and apex	approx. midway between base and apex	closer to apex	slightly closer to base
sculpture	numerous spiral striae	4 - 9 strong spiral ribs	numerous spiral striae	4 - 9 weak spiral ribs, or spiral striae	10 - 14 weak to strong spiral ribs, or fine spiral striae	10-14 weak to strong spiral ribs, often nodulate; early whorls cancellate	numerous spiral striae	more than 14 weak spiral ridges, often paired; early whorls weakly cancellate
depression below suture	moderate	moderate to strong	slight to moderate	moderate to strong	slight to none	slight to moderate	slight to none	slight
profile of upper whorls	slightly convex	straight to stepped	slightly convex	straight to stepped	distinctly convex	slightly convex	straight	straight

(cont)

Table 1 continued.

Features	species							
	B. catarrhacta	*B. c. cincta*	*B. c. limbosa*	*B. lagenaria*	*B. papyracea*	*B. pubescens*	*B.* sp. A	*B.* sp. B
interior of aperture	aperture smooth, crenulated at margin	aperture smooth, undulated at margin	aperture plicate	aperture smooth, crenulated at margin	aperture plicate	aperture plicate	aperture plicate	aperture plicate
parietal scar	dark	pale	pale	dark	pale	pale	dark	pale
periostracum	thin, yellowish-brown	thick, fibrous greenish-brown	thick, fibrous brown	thick, fibrous greenish-brown	thin, papery, yellowish-brown	thin, papery, yellowish-brown	thick, fibrous greenish-brown	thin, yellowish-brown
shell colour	greenish- to yellowish-brown with brown axial flames, occ. brown	dark brown, occasionally with a few pale flecks	dark brown	yellowish-brown to brown with dark and pale dashes, or brown	reddish-brown to pale brown	yellowish- to reddish-brown to pale brown, often flecked	dark bluish-brown with pale spiral bands	orange- to reddish-brown, with flecks only on early whorls
aperture colour	dark brownish-violet	pale mauve-brown to violet	white to pale mauve-brown	dark brownish-violet	white to pale mauve-brown	white to pale mauve-brown	pale white-mauve, or dark brown-violet	white to pale mauve-brown
live specimens with bryozoan	no	no	no	no	yes	yes	no	yes
colour of proboscis	black	flesh coloured	flesh coloured	flesh coloured	flesh coloured	flesh coloured	flesh coloured	flesh coloured
shell length (mm)	21 - 35	32 - 64	30 - 60	19 - 45	23 - 58	21 - 41	27 - 46	34 - 46

populations, a finding which backed up the proposal that they are subspecies. Using a key that was based on a combination of loci and species-specific alleles, all of the individuals of most of the species could be identified. Only in the case of *B. cincta* and *B. lagenaria*, was it not possible to unambiguously separate all of individuals, but even in this case the probability of making the correct assignment was high (above 95%).

Using the information provided by both the morphometric and electrophoretic data, I distinguish seven species, one being divisible into two subspecies. In the present Chapter I have provided descriptions of these and a key to the species. In conjunction with remarks furnishing details of particular characters (or suites of characters) which can be used to distinguish between morphologically similar species, this should enable one to correctly identify the majority of the individuals belonging to this genus. However, within species, the form and colour of the shell can be influenced by local environmental conditions, giving rise to differences between local populations. Unquestionably, there will always some individuals which will either be incorrectly identified, or will not be identified with any certainty.

A summary of the typical features for each of the species of *Burnupena* is given in Table 1. The most significant features (or combination of features) diagnosing each of the species are as follows:

B. catarrhacta: relatively high spired, pattern of axial flames on the shell, dark parietal scar and black proboscis.

B. c. cincta: sculptured by four to nine strong spiral ribs.

B. c. limbosa: sculptured by numerous fine spiral striae, aperture plicate internally.

B. lagenaria: low spired, squat, strongly depressed below the suture.

B. papyracea: profile of upper whorls distinctly convex, little to no depression below the suture, periostracum papery.

B. pubescens: early whorls cancellate, 10 to 14 spiral ribs, shell with flecked pattern.

B. sp. A: low spired, squat, sculptured by numerous fine spiral striae, little to no depression below the suture.

B. sp. B: early whorls weakly cancellate, more than 14 weak spiral ridges, shell with flecked pattern on upper whorls only with rest of shell a uniform colour.

In addition to the seven living species of *Burnupena* that have been identified, two fossil species from Hondeklip (on the west coast) have been described (Kensley & Pether, 1986). The first of these, *B. rogersi*, was described as follows: shell plump, about 1.5 times longer than wide; profile of the early whorls evenly convex; sculptured by fine spiral bands; strongly sunken suture; inner surface of outer lip with faint ridges. Kensley and Pether noted that this species bore some resemblance to the genus *Babylonia*, but remarked that the South African species of the *Babylonia* do not possess spiral sculpture. They also noted that *B. rogersi* resembled some species of *Burnupena*, especially *B. papyracea*, which does sometimes have a sunken suture. They note however, that the generic position cannot be established with certainty in the absence of the radula, soft parts and operculum. The second fossil species, *B. aestus*, was described as follows: shell elongate, slender, spire longer than aperture; profile of whorls convex with slight depression below the suture; sculptured by fine spiral lirae; inner surface of outer lip ridged. In their remarks, Kensley and Pether noted that this species most resembles *Afrocominella capensis* in general proportions, but lacks the axial ridges of the early whorls typical of this species. *B. aestus* is also similar to *B. papyracea*, although this species is not as slender and has fewer spiral lirae.

The results of this and the previous three Chapters permit an assessment of the number of valid species, and the characteristics by which they can be identified. Several of these species would have been difficult or impossible to detect purely on morphological grounds, but were clearly distinct when analysed electrophoretically. One might expect that those species which are difficult to differentiate morphologically, or are electrophoretically similar, should be most closely related. The following Chapter is concerned with elucidating such phylogenetic relationships between the species of *Burnupena*.

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Appendix A

Case 0000

***Buccinum cinctum* Röding, 1798 (Mollusca, Gastropoda, Buccinidae): proposed conservation of the specific name.**

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Abstract. The purpose of this application is to conserve the specific name of the South African whelk *Burnupena cincta* (Röding, 1798). This species is very abundant and has been widely studied taxonomically and ecologically. The specific name *Buccinum mexicanum* Bruguière, 1789 is a senior objective synonym of *Buccinum cinctum*, but it has never been used subsequent to the original description. Conversely, the species has been frequently referred to under the name *Burnupena cincta*. Suppression of the name *Buccinum mexicanum* Bruguière, 1789 is proposed.

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1. *Buccinum mexicanum* Bruguière (1789, p. 260), was originally reported as occurring on the Mexican coast. No illustration was given, but Bruguière listed in his synonymy the work of Chemnitz (1780, p. 71, pl. 126, figs. 1213,1214). However, he noted that although Chemnitz's non-binomial shell was most probably the same species as *Buccinum mexicanum*, it was difficult to compare them as Chemnitz's drawings were not very accurate. Nevertheless, he concluded that the two species were not different. The given locality was clearly inaccurate as the taxon is now known to be endemic to South Africa.

2. Gmelin (1791, p. 3494) proposed the name *Buccinum porcatum* for Chemnitz's figures, but although this name was used extensively until the early part of this century, Iredale (1918, p. 34) pointed out that it was a junior homonym of *Buccinum porcatum* da Costa, 1778.

3. Röding (1798, p. 113) based the name *Buccinum cinctum* on a specimen in Bolten's collection, which is now unrecognisable, but he gave references to the same figures in Chemnitz (1780), and also referred Gmelin's *Buccinum porcatum* to the same species.

4. *Buccinum mexicanum* was listed as a synonym of *Buccinum porcatum* by Dillwyn (1817, p. 635) and Reeve (1846, pl. 4, sp. 22), neither of whom commented on the priority of the former name. Deshayes (1844, p. 190) similarly listed *Buccinum mexicanum* as a synonym of *Buccinum porcatum*, and remarked that Bruguière described this species but was not aware of Gmelin's name which had precedence. However, Bruguière's name predates that of Gmelin.

5. Lamarck (1822, p. 244) described *Purpura ligata*, but under this species Deshayes (1844, p. 78) listed as synonyms *Buccinum mexicanum* and *B. porcatum*, and in a footnote, commented that this species was a *Buccinum* not a *Purpura*, and that Lamarck did not know of Gmelin's work.

6. Gray (1850, p. 72) erected the genus *Cominella* and transferred *Buccinum ligatum*, along with other species, to it. Kobelt (1878, p. 231) placed *Buccinum porcatum* in the genus *Cominella*. Iredale (1918, p. 34) proposed the genus *Burnupena*, and referred various South African "*Cominella*" species, including *B. porcatum*, to this genus on the basis of both shell and radular characters. He designated *Buccinum cinctum* as the type species for the genus.

7. The name *Buccinum mexicanum* has not been used as a senior synonym since its original proposal in 1789. On the other hand, *Buccinum cinctum* (as *Burnupena cincta*) is in common use, and the Commission Secretariat has been given a list of 22 works (11 from the last 35 years) which use this name. These references include Stephenson (1948), Orr (1956), Barnard (1959), Kilburn & Rippey (1982) and Branch *et al.* (1994). The case for the conservation of *Buccinum cinctum* clearly meets the *prima facie* criteria mentioned in Article 79c of the Code.

8. The International Commission on Zoological Nomenclature is accordingly asked, in the interests of stability:

(1) to used its plenary powers to suppress the specific name *mexicanum* Bruguière, 1789, as published in the binomen *Buccinum mexicanum*, for the purposes of the Principle of Priority but not for those of the Principle of Homonymy;

(2) to place on the Official List of Specific Names in Zoology the name *cinctum* Röding, 1798, as published in the binomen *Buccinum cinctum*;

(3) to place on the Official Index of Rejected and Invalid Specific Names in Zoology the name *mexicanum* Bruguière, 1789, as published in the binomen *Buccinum mexicanum* and as suppressed in (1) above.

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Chapter 5

Phylogenetic analysis of the genus Burnupena using allozyme and morphometric data

INTRODUCTION

In this section of my study, I formulate a phylogenetic hypothesis for the species of *Burnupena*. Thus far, there has been only one preliminary attempt to elucidate the relationships between five of the species (Grant et *al.*, 1988). This study indicated that *B. catarrhacta* and *B. papyracea* are genetically distinct from *B. cincta* , *B. limbosa* and *B. lagenaria*, and that *B. catarrhacta* is more basal, having diverged from the other species 5 to 10 million years ago, whereas the evolutionary divergence between the other species was much more recent. The evolutionary relationships between *B. cincta*, *B. limbosa* and *B. lagenaria* were, however, unresolved.

There have been several controversies relating to the estimation of phylogenies, concerning both philosophical and methodological issues. One of the theoretical issues is the 'molecules versus morphology' debate, which revolves around whether molecular or morphological data are better sources of information for estimating phylogeny (Crowe, 1988; Moritz & Hillis, 1990). The advantages of each technique, and the conflicts that have arisen in phylogenetic studies, have been reviewed by Hillis (1987). Briefly, among the advantages of molecular data are the potential size of the data set, the ability to obtain phylogenies not only from recent times but also from ancient times (depending upon which molecules are examined), and the fact that in general, molecular data have a clear genetic basis and are less prone to environmental influences than morphological data. Advantages of using morphology are that data can be obtained from extensive museum collections, as well as from fossils, that ontogenetic information can be used to polarize characters, and that the cost of collecting morphological data is usually much lower. However, Moritz and Hillis (1990) note that the conflicts between molecular and morphometric studies are no greater than those among studies using one technique only. The conclusions drawn are that each type of data has its uses, and that studies incorporating both types of data can provide stronger evidence for the phylogenetic hypothesis if the results from different data sets are congruent.

Another controversy that has been well documented concerns the phenetic versus cladistic (or so called distance versus character data) approaches to phylogenetic systematics. A review of both approaches as they apply to electrophoretic data is given by Buth (1984). The aims of phenetics, stated in Sneath and Sokal (1973) are "the numerical evaluation of the affinity or similarity between taxonomic units and the ordering of these units into taxa on the basis of their affinities". A single measure of overall similarity or distance is calculated for each pair of taxa, and the similarity matrix obtained is then used to construct a tree, referred to as a phenogram, which indicates the relationships between the taxa. Although there are several measures of genetic distance and similarity, as well as clustering algorithms, those commonly employed are Nei's (1978) and Rogers' (1972) distances, the unweighted pair-group method with arithmetic averages (UPGMA - Sneath & Sokal, 1973), and the distance Wagner (Farris, 1972) algorithms. One problem with the UPGMA technique (that does not apply to the distance Wagner procedure) is that it assumes a constant rate of divergence for all taxa, a condition all too often unsatisfied in real data sets (Baverstock et *al.*, 1979; Swofford, 1981). This can lead to possible errors in interpreting phylogenies from phenetic analyses. Criticisms of the phenetic approach include the following: (1) many different topologies of phenogram can be generated depending upon the measure used to create the similarity matrix and the clustering algorithm chosen, thereby negating one of the aims of phenetics, namely objectivity; (2) the use of overall similarity to group taxa does not take into consideration problems of character convergence, although pheneticists argue that if enough characters are used, the affect of these characters will be minimal; (3) taxa are grouped on the basis of the presence as well as the absence of characters, which can lead to the clustering of taxa simply because they do not have a character that is present in other taxa (Scotland, 1992). Although distance methods have been widely criticised, they are still commonly used for estimating phylogenies, one of the reasons being that genetic distance may be correlated with time since divergence (Crowe, 1988). This relationship forms the basis of the molecular clock hypothesis (Thorpe, 1982; Thorpe & Solé-Cava, 1994). Although there is wide acceptance that a broad correlation exists between the amount of molecular divergence and evolutionary time, there is considerable disagreement over the constancy of divergence rates and the usefulness of molecular data to predict times of divergence with any accuracy (Hillis & Moritz, 1990). Proponents argue that

a "sloppy" clock is better than nothing, whilst critics maintain that there are too many sources of error and that the results are highly misleading.

On the other hand, the cladistic approach, most coherently formulated by Hennig (1966), makes use of discrete character data rather than reducing the data to a single measure. Furthermore, although Hennig recognised that similarities are important, not all types of similarity are phylogenetically informative. He differentiated three types of characters: (1) plesiomorphic characters are those thought to have arisen amongst the ancestors of the group of organisms under study, and are deemed to be primitive or ancestral; (2) apomorphic characters are found only in the group they define, and are regarded as derived or advanced; (3) homoplasic characters are those which have lead to similarities in character state for reasons other than inheritance from a common ancestor, such as convergence, parallelism and reversals. In the cladistic approach, phylogenies are constructed using only shared-derived characters or synapomorphies. Shared primitive characters (symplesiomorphs) and characters unique to a single taxon (autapomorphs) provide no evidence of phylogenetic relationships. The determination of character polarity, whether it is primitive or derived, is generally accomplished using one of two criteria, namely ontogeny and outgroup comparisons, although the use of ontogenic information is restricted to morphological data (Hillis, 1987). The outgroup comparison method, as defined by Watrous and Wheeler (1981), is that "for a given character with two or more states within a group, the state occurring in related groups is assumed to be the plesiomorphic state". The presence of homoplasies in the data can lead to conflicts between the characters, and can result in the production of more the one cladogram. In such cases, the number of conflicts are minimized by employing the parsimony principle, which minimizes the number of evolutionary steps required to explain the data. In this way the parsimony criterion maximizes character congruence, and minimizes homoplasies (Swofford & Olsen, 1990; Scotland, 1992). Disadvantages of the cladistic approach to phylogenetic systematics are as follows (Patton & Avise, 1983): (1) for a given data set a large number of equally parsimonious trees can be generated; (2) only a small percentage of the electrophoretic characters contribute to the identification of clades (although cladists argue that these are the only phylogenetically informative characters); (3) rare alleles carry as much weight as those that are abundant; (4) the analyses are

critically dependent on the choice of the outgroup taxa which determines the polarity of the characters; (5) the evolutionary relationships of the alleles at a given locus are not readily apparent.

With regard to debates involving methodological issues, for both phenetic and cladistic approaches, a number of different methods have been proposed for treating the data (calculating distances or coding the data), as well as for the production of trees (phenograms or cladograms). For both types of analysis, the choice of methods can influence the results. Swofford and Olsen (1990) noted that a potential weakness of using electrophoretic data in cladistic analysis is the lack of a universally accepted method for the construction of phylogenies from these data. A review of selected studies by Mabee and Humphries (1993) revealed that most investigators used more than one method of coding their electrophoretic data, and that phenetic methods were also commonly employed. Emberton (1994a) noted that in molluscan systematics, distance methods were by far the most commonly used, and that cladistic methods were relatively uncommon.

The objectives of this section of the thesis were to formulate phylogenetic hypotheses for the species of *Burnupena*, using both morphological and allozyme data for the construction of evolutionary trees. For the allozyme data, different coding methods for the cladistic analysis will be utilised. A phenetic analysis of the allozyme data will also be performed, and the resulting phenograms compared to the cladograms produced. The trees obtained from the analysis of the allozyme data set can thus be compared with those obtained from the morphological data. Any congruence arising from the independent data sets will obviously increase confidence in a particular phylogeny.

METHODS

MATERIALS

Data for the phylogenetic analyses were derived from the morphometric (see Chapter 1) and electrophoretic (Chapter 3) analyses. Data for all populations of each species were pooled, but the two subspecies of *B. cincta* (*cincta* and *limbosa*) were analysed separately because they have previously been regarded as separate species. In addition, data were collected for *Afrocominella capensis*, a species belonging to a closely related genus, which was used as the outgroup taxon. Twenty nine individuals of this species were collected from A-Frame on two occasions over two months during 1990, and these specimens were electrophoresed together with representatives of all of the species of *Burnupena*. Table 1 lists the species, the abbreviations used, and the number of animals used for each of the data sets.

ALLOZYME DATA

Treatment of the allozyme data employed both distance and character data to allow a comparison of the different approaches. Rogers' modified distance (*D* - Wright, 1978) was used for the phenetic analysis. Genetic distances were calculated for all pairwise comparisons of the species, and the matrix used in the distance Wagner procedure (Farris, 1972) to construct a tree using *A. capensis* as the outgroup to root the tree. Swofford's (1981) multiple addition criterion and the goodness-of-fit criterion (*F*-value) of Prager and Wilson (1976) were employed. The Rogers' distance is used here because it satisfies the Wagner requirement of triangle inequality. The distance Wagner procedure is used because phylogenetic relationships between taxa can be inferred, since this procedure does not assume constant evolutionary rates. However, the tree should not then be rooted using midpoint rooting, which does require the assumption of equal rates of divergence among lineages (Hillis, 1987).

Table 1. List of species of *Bumupena* and *Afrocominella*, abbreviations used, and sample sizes for the allozyme and morphometric analyses.

		sample sizes:	
Species	abbrev.	allozyme	morphology
B. c. cincta	CIN	231	207
B. c. limbosa	LIM	106	79
B. papyracea	PAP	194	181
B. pubescens	PUB	78	75
B. lagenaria	LAG	164	215
B. sp. A	A	68	59
B. sp. B	B	19	16
B. catarrhacta	CAT	47	40
A. capensis	CAP	28	29

Although cladistic analysis of morphological characters has been used successfully for many groups, cladistic analysis of allozymes for the purposes of constructing phylogenies is more recent (reviewed by Buth, 1984; Swofford & Olsen, 1990). Two fundamental problems with the coding of electrophoretic data have arisen. Firstly, what unit constitutes the character, and secondly, how the character states are ordered. A number of methods have been proposed for the coding of such data (reviewed by Buth, 1984; Mabee & Humphries, 1993). One approach, known as the *independent alleles* model (Mickevich & Mitter 1981), is to treat the allele as the character, with the states being either present or absent. However, criticisms levelled at this method (Mickevich & Mitter 1983; Buth, 1984; Swofford & Olsen, 1990) are that the alleles at a single locus are not independent, an assumption made in most methods of analysis, and that consequently, it is biologically unrealistic since hypothetical ancestors may contain no alleles for some loci.

An alternative approach is to use the locus as the character, with the alleles or allelic combinations as the states. This raised the problem of how to order the character states. Mickevich and Mitter (1981, 1983) proposed four methods for inferring character state order; the *shared alleles* model, the *minimum allele turnover* model, the *relative mobility* model and a *systematic* method. Each of these methods makes different assumptions regarding the theories of allozyme evolution.

A method that has recently become available as an option in computer algorithms (PAUP version 3.0 (Swofford, 1989)) for the coding of polymorphic data such as electrophoretic data, employs the use of a step matrix, which allows the input of complex branched or reticulate character state trees (Mabee & Humphries, 1993). A step matrix is a character-state by character-state distance matrix in which the distances between the states reflect the number of evolutionary steps between each state. This option retains the biologically more realistic definition of the locus as the character, but without the loss of character state homology from shared allele combinations that results when the states are ordered into linear or branched character state trees, a merit shared with the *independent alleles* model where the allele is considered to be the character (Mabee & Humphries, 1993).

Another debatable issue concerning coding of electrophoretic data, involves the importance of allele frequency information. Buth (1984) distinguished between "qualitative" coding, in which the alleles are regarded either as present or absent irrespective of their frequency, and "quantitative" coding where differences in allele frequency are deemed to be informative and as such are recognised as separate states. Both types of coding can be applied to methods using the allele as well as the locus as the character. Criticisms have been levelled at both types of coding. Critics of the use of frequencies (Farris, 1981; Mickevich & Mitter 1981, 1983) maintain that frequencies are not useful, and that in most cases (subspecies or higher), pairs of taxa differ either almost completely or show similar allele frequencies for any given locus. Further, frequencies can be modified by processes such as gene flow, selection and genetic drift, and if there is frequency variation among populations, then the frequencies for the species may be difficult to estimate (Mabee & Humphries, 1993). On the other hand, proponents of the use of frequencies (Swofford & Berlocher, 1987; Swofford & Olsen, 1990) claim that qualitative coding fails to address the possibility that alleles present at low frequencies may not be detected if the sample size is small. Swofford and Berlocher (1987) found that frequency estimates were less susceptible to small-sample problems, and contend that frequencies provide a means to weight the presence or absence of alleles, with those occurring at low frequencies given less importance than those fixed or present at high frequencies.

In this study, frequency information was not used, since allele frequencies of the populations within species varied depending on the locality of the population. Three different approaches were employed. Firstly, the *independent allele* model (Mickevich & Mitter, 1981) was used where the allele is the character, and its presence or absence in a taxon are the character states. Secondly, the locus was treated as the character and the alleles were scored as present or absent. Allelic combinations at each locus were the character states, and these were ordered using the *minimum allele turnover* model (Mickevich & Mitter, 1983). Under this model shared alleles as well as losses are regarded as significant, and states are connected to minimize the total number of allelic changes. Branched character state trees were coded using the ordinal coding method described in Mickevich and Weller (1990). The third method employed also treated the locus as the

character, but the step matrix option in PAUP was used to reflect the hypotheses of character homology. In all three methods the outgroup comparison method (Watrous & Wheeler, 1981) was used to polarize the characters, whereby the allele or allelic combination present in the outgroup taxon was assumed to be the plesiomorphic state. Characters for which the gels could not be scored were coded as unknown.

MORPHOLOGICAL DATA

The use of continuous morphological data are not commonly employed in cladistic analyses (Thiele, 1993). Detractors maintain that such data do not contain cladistic information, that branching diagrams are unsuitable for this type of data, and that any phylogenetic signal present cannot be separated from the 'noise" of such data, and that therefore, continuous data are best excluded from analysis (Pimental & Riggins, 1987; Cranston & Humphries, 1988). Chappill (1989) argues that, although continuous characters are more noisy than discrete characters, they can be used when the number of qualitative characters are not sufficient for the resolution of phylogenetic relationships, but cautions that resulting phylogenies should be regarded as provisional. Further, until the methods proposed by Archie (1985), and more recently by Thiele, (1993), there were few techniques which provided a means for coding continuous data into a form suitable for analysis in cladistic programs. However, Thiele (1993) maintains that continuous morphometric data should not be excluded a *priori* from cladistic analyses, unless it can be shown empirically that they contain no or very little phylogenetic information, or that the method of cladistic analysis used cannot handle such data.

In this study, both the quantitative and qualitative variables described in Chapter 1 were employed, despite reservations about the suitability of quantitative data for cladistic analysis. Due to the strong correlation between the shell measurements, the quantitative morphological data were standardised by using ratios of the various shell measurements, as described in Chapter 1, as the characters for the cladistic analysis. Shell length was also used to represent overall size. These data

were coded using the gap-weighting procedure described by Thiele (1993). The means for the species were log transformed to stabilise the variance, then range standardized using the minimum and maximum means values:

$$x_s = \frac{\bar{x} - \min}{\max - \min}$$

Although Thiele then multiples the value obtained (x_s) by the maximum number of states allowable by the computer algorithm used (10 in the case of Hennig86), I have reduced the possible number of states to five, since there are only nine taxa (including the outgroup). The values obtained were then coded to the rounded integer of their standardized values, which were then treated as ordered multistate characters.

Four of the qualitative variables used in Chapter 1, namely lip constriction, aperture sculpture, parietal scar and spire angle, were treated in the same manner as the quantitative variables (coded using gap-weighting and treated as ordered multistate characters) due to the presence of more than one state in most of the species (see Chapter 1, Fig. 5, showing the frequency histograms of the qualitative variables). The other two qualitative variables, rib strength and the number of ribs were not coded in this way, since the means would give misleading results due to the bimodal distribution of the states for *B. papyracea*. Instead, the states for these two characters were identified as follows:

- Rib strength: ribs absent (0), ribs present and weak (1), ribs present and strong (2). One of the taxa, *B. cincta cincta* was coded as having an unknown state since approximately 25% of the individuals had weak ribs, and the remaining individuals had strong ribs.
- Number of ribs: ribs absent (0), if ribs present and few (1), intermediate (2), many (3).

These two characters were treated as unordered multistate characters.

In addition to the characters used in the morphometric analyses, three additional characters, described below, were used in the phylogenetic analysis.

- Aperture colour: white to pale (0), dark (1).
- Axial ribs: absent (0), present (1).

- Shell covered by bryozoan: absent (0), present (1).

DATA ANALYSIS

The phenetic analyses were performed using the BIOSYS-1 computer program (release 1.7) of Swofford and Selander (1981). Cladistic analyses were performed using Hennig86 (Farris, 1988), and PAUP (version 3.0, Swofford, 1989). Hennig86 was employed for both the morphological and allozyme data matrices, using the implicit enumeration (ie) option for calculating the trees. The consistency (Kluge & Farris, 1969) and retention (Farris, 1989) indices were used to measure the fit of the data to the phylogenetic trees, and were calculated excluding the autapomorphies. All characters were unweighted. When the analyses yielded more than one cladogram, a strict consensus tree was calculated using the nelsen option. PAUP was employed for the allozyme data using the step matrix option to code the characters. A heuristic search was performed using the MULPARS option.

Table 2. Matrix of Rogers' Modified genetic distance coefficients for the species of *Burnupena* and *A. capensis*. Abbreviations as in Table 1.

Species	CIN	LIM	PAP	PUB	LAG	A	B	CAT	CAP
B. c. cincta	*****								
B. c. limbosa	0.09	*****							
B. papyracea	0.37	0.38	*****						
B. pubescens	0.49	0.49	0.49	*****					
B. lagenaria	0.26	0.24	0.42	0.51	*****				
B. sp. A	0.43	0.45	0.44	0.50	0.47	*****			
B. sp. B	0.41	0.41	0.40	0.47	0.48	0.32	*****		
B. catarrhacta	0.81	0.81	0.82	0.79	0.82	0.84	0.82	*****	
A. capensis	0.87	0.87	0.87	0.84	0.88	0.89	0.89	0.92	*****

RESULTS

ALLOZYME DATA

Of the 25 loci scored for *Burnupena*, two (*AAT-2* and *ME-1*) could not be scored for *A. capensis*. Ten of the loci were monomorphic for *A. capensis*, with only two of these having an allele that is also present in *Burnupena*. The remaining 13 loci were polymorphic, five of which shared no alleles with *Burnupena*, whereas the other eight had 13 alleles which were common to both genera. The allele frequencies of the polymorphic loci for all species are given in Appendix A.

Phenetic analyses

Rogers' modified distance (Wright, 1978) was calculated for all pairwise comparisons (Table 2), and the distance matrix was used by the distance Wagner procedure (Farris, 1972) to construct a tree using *A. capensis* as the outgroup to root the tree. Two trees were produced, which differed only in the placement of *B. papyracea*. This species was either joined to the *B. cincta* - *B. lagenaria* cluster or to the *B.* sp. A - *B.* sp. B cluster. In all other respects, the two trees were identical. Of the two trees produced, the one presented (Fig. 1) had slightly better goodness-of-fit statistics, although this does not imply that this tree is more accurate. Interestingly, the dendrogram produced using Nei's genetic distance and the UPGMA clustering algorithm, had the same topology as the tree presented here.

Examination of the Wagner tree (Fig. 1) confirms the close genetic relationship between the two subspecies *B. cincta cincta* and *B. cincta limbosa*. Also, as expected from the results of the previous Chapter, *B. catarrhacta* was genetically the most distant species. Different branch lengths can reflect different rates of change (Felsenstein, 1984), but may also reflect length of time since separation. The very long branch of *B. catarrhacta* probably reflects the latter since the distance of all of the species to the root is roughly the same, suggesting nearly equal rates of change along all branches.

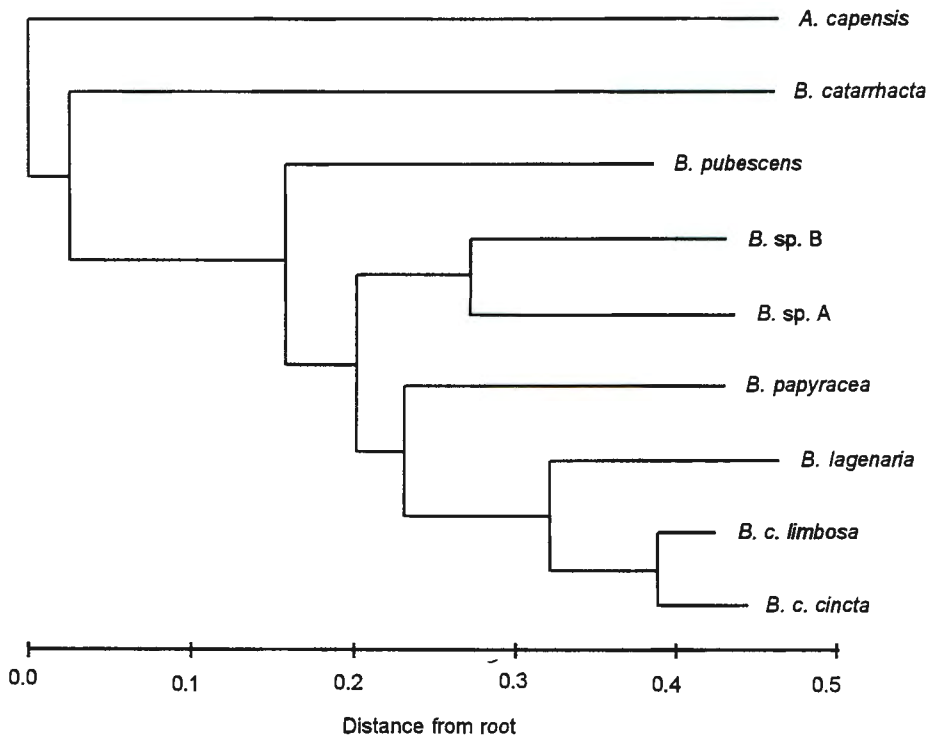

Figure 1. A Wagner tree constructed using Rogers' distance indicating the relationships between the species of *Burnupena*. The tree was rooted using *A. capensis* as the outgroup. Total tree length = 2.32, Prager and Wilson's (1976) "F-value" = 2.39, cophenetic correlation = 0.997.

Cladistic analyses

Of a total of 130 alleles detected, 28 alleles were unique to *A. capensis*, and 20 were unique to *B. catarrhacta* (Appendix A). Only 60 alleles proved to be phylogenetically informative and were used as the characters in the first analysis using the *independent allele* model. The distributions of the characters among the species are given in Table 3. An initial analysis yielded two equally parsimonious cladograms, each with length (L) 105, consistency index (CI) of 0.56 and retention index (RI) of 0.47. The only difference between them was whether *B. cincta cincta* formed a clade with *B. cincta limbosa* (supported by three homoplasies), with *B. lagenaria* as the sister species, or, whether *B. c. limbosa* and *B. lagenaria* formed a clade (supported by a single homoplasy) with *B. c. cincta* as the sister species. The former tree, showing the two subspecies of *B. cincta* as closest relatives is presented in Fig. 2. The results show that the genus is monophyletic, this being supported by a number of synapomorphies. As with the Wagner tree, *B. catarrhacta* is placed as the sister species to all other *Burnupena* species, which form a monophyletic group supported by a large number of synapomorphies. The close relationship between the two *B. cincta* subspecies and *B. lagenaria* is evident, although this cluster is only supported by one synapomorphy. *B. papyracea* and *B. pubescens* are shown as sister species, supported by a large number of synapomorphies, and these together with *B. c. cincta*, *B. c. limbosa* and *B. lagenaria* form a monophyletic clade. The main difference between this tree and the Wagner tree is the placement of *B. pubescens*.

When the locus was used as the character, and the states ordered using the *minimum allele turnover* model, a total of 41 characters was derived from the 25 loci examined, although 18 of these were autapomorphs. The data matrix of the allozyme character states (including the autapomorphs) for all of the species is given in Table 4. The initial analysis using only the 23 informative characters, yielded two equally parsimonious cladograms, each with a length (L) of 118, a consistency index (CI) of 0.65 and a retention index (RI) of 0.43. The only difference between them was the placement of *B.* sp. A and *B.* sp. B. The strict consensus tree (L = 120) derived from the

Table 3. Data matrix for the analysis using the allele as the character, showing the presence / absence of the characters for each of the species. All autapomorphs have been removed. Species abbreviations are as in Table 1.

Locus/allele		character number	CIN	LIM	PAP	PUB	species LAG	A	B	CAT	CAP
ARK	32	1	0	0	0	1	0	0	0	0	1
	52	2	1	1	1	1	1	1	1	0	1
	70	3	1	1	1	1	1	1	1	0	0
	100	4	0	0	1	0	0	1	0	0	0
AAT-1	-100	5	1	1	1	1	1	1	1	0	0
	-65	6	0	0	1	1	0	0	0	0	0
AAT-2	65	7	1	1	1	1	1	1	1	0	?
	100	8	1	1	1	1	1	1	0	1	?
	127	9	0	0	0	0	0	0	0	1	?
DIA-1	94	10	0	0	1	1	1	1	1	1	0
	100	11	1	1	1	0	1	0	1	0	0
G6PDH	100	12	1	1	1	1	1	1	1	0	1
GPI	100	13	1	1	1	1	1	1	1	0	0
	315	14	1	0	1	1	0	0	1	0	0
GAP	-100	15	1	1	1	1	1	1	1	0	1
HEX-3	95	16	0	0	1	1	0	0	0	0	0
	98	17	0	0	1	0	1	1	0	0	0
	100	18	1	1	1	1	1	1	1	0	0
IDH-1	10	19	1	0	1	0	0	0	0	0	0
	100	20	1	1	1	1	1	1	1	1	0
	236	21	1	1	1	1	1	1	0	0	0
IDH-2	83	22	1	0	0	1	1	0	1	0	0
	100	23	1	1	1	1	1	1	1	1	0
	117	24	0	0	0	1	1	0	0	0	0
LAP	100	25	1	1	1	1	1	0	0	0	0
	103	26	0	0	0	0	0	1	1	0	0
	106	27	1	1	0	1	1	0	0	0	1
	109	28	1	1	0	1	1	0	0	0	1
LDH	100	29	1	1	1	1	1	1	1	1	0
MDH-1	-32	30	0	0	1	1	0	0	0	0	0
	100	31	1	1	1	1	1	1	1	0	0
MDH-2	100	32	1	1	1	0	1	1	1	0	0
MDH-3	100	33	1	1	1	1	1	1	1	0	0
ME-1	100	34	1	1	1	1	1	1	1	1	?
ME-3	100	35	1	1	1	1	1	1	1	0	1
MPI	75	36	0	1	0	1	1	0	0	0	0
	88	37	1	1	0	0	0	1	1	0	1
	97	38	1	1	0	1	1	1	0	0	1
	100	39	0	0	1	0	1	0	1	0	1
	106	40	1	1	1	0	1	0	1	0	1
ODH	62	41	1	1	0	0	0	0	1	0	0
	82	42	1	1	0	0	1	0	0	0	0
	100	43	1	1	1	1	1	1	1	0	0
	115	44	0	1	1	1	0	0	0	0	1
GL	100	45	1	1	1	0	1	1	1	0	1
	111	46	0	0	1	1	0	1	1	0	0
	115	47	1	1	0	0	1	1	0	0	0
PHP	100	48	1	1	1	1	1	1	1	0	0
	104	49	0	0	0	1	1	0	0	0	0
PGM	69	50	1	1	1	0	1	0	0	1	0
	86	51	1	1	1	0	1	0	0	1	0
	100	52	1	1	1	0	1	1	1	0	1
	111	53	1	1	1	1	0	1	1	0	0
	121	54	0	0	1	1	0	0	0	0	0
PGD	-455	55	0	0	1	0	0	1	0	0	1
	-182	56	0	0	0	1	0	0	1	0	0
	-100	57	1	1	1	1	1	0	1	0	0
SDH	100	58	1	1	1	1	1	1	1	0	0
	106	59	1	1	1	1	1	0	0	0	0
SOD	100	60	1	1	1	1	1	1	1	1	0

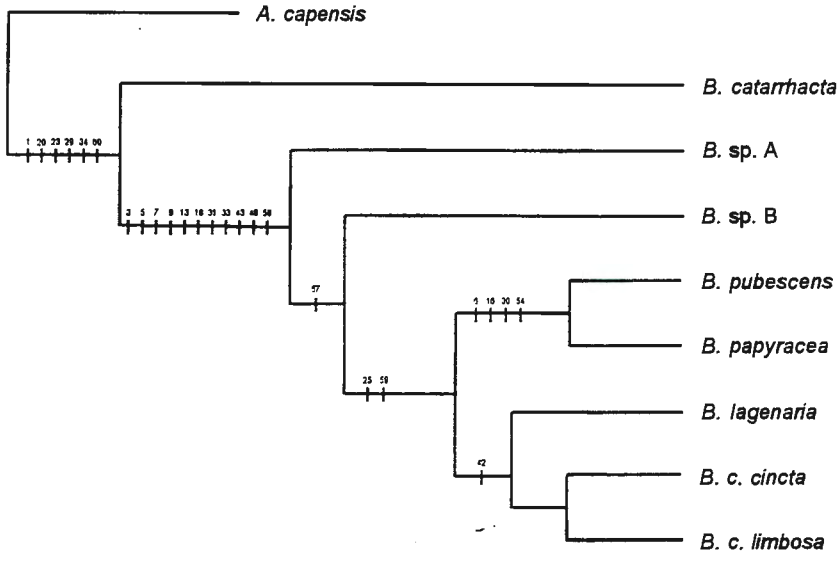

Figure 2. Cladogram produced from the analysis using the allele as the character, illustrating the hypothesised relationships among the species of *Burnupena*. Bars indicate synapomorphies with the numbers referring to the character. The *B. c. cincta* and *B. c. limbosa* grouping is supported by three homoplasies (characters 39, 41 and 53). In the other equally parsimonious tree produced, the *B. lagenaria* and *B. c. limbosa* grouping was supported by a single homoplasy (character 36).

Table 4. Data matrix for the analysis using the locus as the character with the states ordered using the minimum turnover model, showing the character states for all of the species of *Burnupena* and *Afrocominella*. Species abbreviations are as in Table 1.

	character	species								
Locus	number	CIN	LIM	PAP	PUB	LAG	A	B	CAT	CAP
ARK	1	2	2	4	1	2	3	2	2	0
	2	0	0	0	0	0	0	0	1	0
AAT-1	3	2	2	3	3	2	2	2	1	0
	4	0	0	0	0	0	0	1	0	0
AAT-2	5	2	2	2	2	2	2	1	3	?
DIA-1	6	3	4	3	1	2	1	2	1	0
G6PDH	7	0	0	0	0	0	0	0	1	0
GPI	8	2	1	3	2	1	1	2	1	0
	9	0	0	0	0	0	0	0	1	0
	10	0	0	0	1	0	0	0	0	0
GAP	11	1	1	1	1	1	1	1	2	0
HEX-3	12	1	1	3	4	2	2	1	0	0
	13	0	0	0	0	0	0	0	1	0
	14	0	0	0	0	1	0	0	0	0
IDH-1	15	3	2	3	2	2	2	1	1	0
IDH-2	16	2	1	1	3	3	1	2	1	0
LAP	17	1	1	2	1	1	3	3	4	0
LDH	18	1	1	1	1	1	1	1	1	0
MDH-1	19	2	2	3	3	2	2	2	1	0
	20	0	0	0	0	0	0	1	0	0
MDH-2	21	1	1	1	2	1	1	1	3	0
MDH-3	22	2	3	2	2	2	2	2	1	0
ME-1	23	1	1	1	1	1	1	1	1	?
ME-3	24	0	0	0	0	0	0	0	1	0
MPI	25	1	2	5	3	4	1	6	3	0
	26	0	0	0	0	0	1	0	0	0
	27	0	0	0	0	0	0	0	1	0
ODH	28	5	6	2	1	4	3	5	3	0
	29	0	0	0	0	0	0	0	1	0
	30	0	0	0	0	0	0	1	0	0
GL	31	1	1	4	5	1	3	4	6	0
	32	1	0	0	0	0	0	0	0	0
	33	0	0	1	0	0	0	0	0	0
PHP	34	2	2	2	3	3	2	2	1	0
	35	0	0	0	0	0	1	0	0	0
PGM	36	2	2	2	3	3	1	1	4	0
	37	0	0	1	2	0	0	0	0	0
PGD	38	3	3	2	5	3	1	4	3	0
	39	0	0	0	0	1	0	0	2	0
SDH	40	3	3	3	4	3	2	2	1	0
SOD	41	1	1	1	1	1	1	1	1	0

two trees is shown in Fig. 3. The positions of *B.* sp. A and *B.* sp. B are shown as an unresolved tricotomy, but the remainder of the tree is fully resolved. The results again show that the genus is monophyletic, and that *B. catarrhacta* is placed as the sister species to all of the remaining *Burnupena* species, with a large number of synapomorphies supporting this latter group. *B. papyracea* and *B. pubescens* again forming a clade, as does *B. lagenaria*, *B. c. cincta* and *B. c. limbosa*, but with *B. lagenaria* the sister to the latter two species, although the *B. c. cincta* - *B. c. limbosa* cluster is not supported by any synapomorphies. These latter five species again form a well supported monophyletic group. This cladogram and that produced with the allele as the character (Fig. 2) are completely congruent, differing only in the areas of resolution. The placement of *B.* sp. A and *B.* sp. B are resolved in Fig. 2, but not in Fig. 3, whilst the positions of *B. lagenaria*, *B. c. cincta* and *B. c. limbosa* are resolved in Fig. 3 but not in Fig. 2.

The third method employed in the cladistic analysis of the allozyme data used a step matrix to order the states of the characters. From the 25 loci, 22 characters were obtained (*G6PDH* and *ME-3* were autapomorphs), and for *ME-1*, the state for *A. capensis* was unknown and this locus was monomorphic for all species of *Burnupena*). The data matrix of the character states for all of the species is given in Table 5. The step matrices for each of the characters are given in Appendix B. The heuristic search found 81 shortest trees, each of length 102. The strict consensus tree is shown in Fig. 4. Although this tree is not well resolved, it does show that the genus is monophyletic. The species were divided into two clades, one with *B. catarrhacta*, *B.* sp. A and *B.* sp. B, and the remaining species forming a separate clade. The members of this latter clade, although not resolved, are those present in the monophyletic group found in the two cladograms produced by the previous methods.

MORPHOLOGICAL DATA

A total of 23 characters was derived from the morphological data (Table 6), of which 18 were coded using gap-weighting and treated as ordered multistate (characters 1 through 14, 20 to

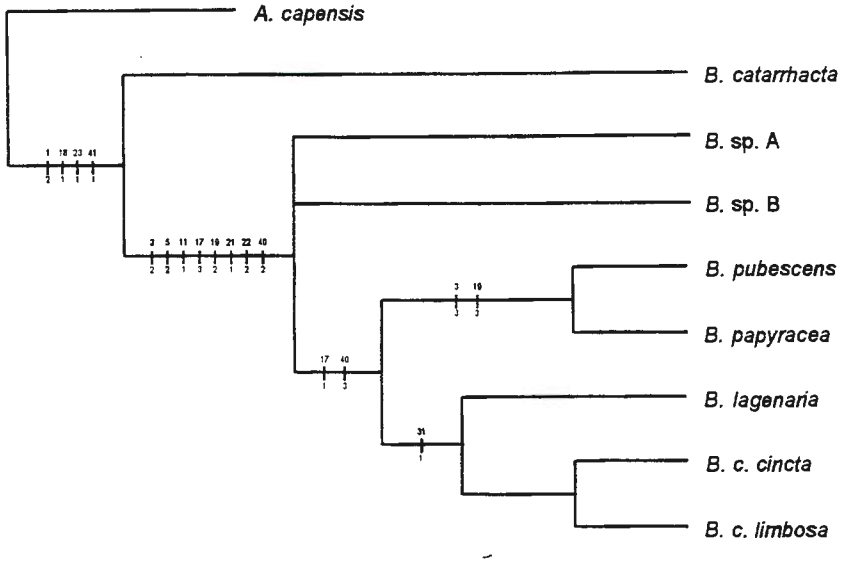

Figure 3. Consensus cladogram produced from the analysis using the locus as the character, and states ordered using the minimum allele turnover model. Bars represent synapomorphies. The numbers indicate the character number (above) and its state (below). The *B. c. cincta* and *B. c. limbosa* grouping is supported by two homoplasies (character 11 - state 1; character 35 - state 2).

Table 5. Data matrix for the analysis using the locus as the character with the states ordered using the step matrix (see appendix B), showing the character states for all of the species of *Burnupena* and *Afrocominella*. Species abbreviations are as in Table 1.

	character	species								
Locus	number	CIN	LIM	PAP	PUB	LAG	A	B	CAT	CAP
ARK	1	1	1	2	3	1	4	1	5	0
AAT-1	2	1	1	2	2	1	1	3	4	0
AAT-2	3	1	1	1	1	1	1	2	3	?
DIA-1	4	1	1	2	3	2	3	2	3	0
GPI	5	1	2	3	4	2	2	1	5	0
GAP	6	1	1	1	1	1	1	1	2	0
HEX-3	7	1	1	2	3	4	5	1	6	0
IDH-1	8	1	2	1	2	2	2	3	3	0
IDH-2	9	3	2	2	1	1	2	3	2	0
LAP	10	1	1	2	1	1	3	3	4	0
LDH	11	1	1	1	1	1	1	1	1	0
MDH-1	12	1	1	2	2	1	1	3	4	0
MDH-2	13	1	1	1	2	1	1	1	3	0
MDH-3	14	1	2	1	1	1	1	1	3	0
MPI	15	1	2	3	4	5	6	7	8	0
ODH	16	1	2	3	4	5	6	7	8	0
GL	17	1	2	3	4	2	5	6	7	0
PHP	18	1	1	1	2	2	3	1	4	0
PGM	19	1	1	2	3	4	5	5	6	0
PGD	20	1	1	2	3	4	5	6	7	0
SDH	21	1	1	1	2	1	3	3	4	0
SOD	22	1	1	1	1	1	1	1	1	0

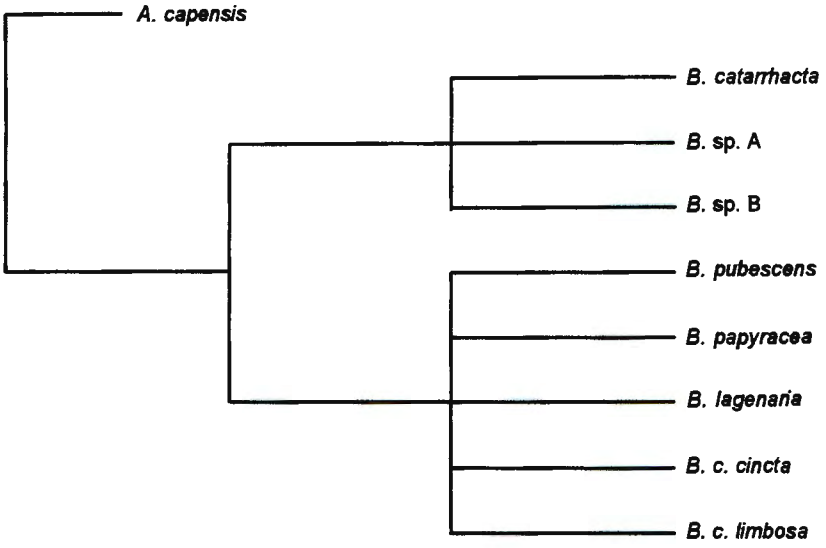

Figure 4. Consensus cladogram produced by using the locus as the character and states ordered using a step matrix.

Table 6. Data matrix for the morphological characters, showing the character states for all of the species of *Burnupena* and *Afrocominella*. Species abbreviations are as in Table 1.

Character	species								
	CIN	LIM	PAP	PUB	LAG	A	B	CAT	CAP
1. Shell width : shell length	2	3	2	2	4	4	2	2	0
2. Spire height : shell length	2	2	4	3	0	1	3	3	4
3. Aperture length : shell length	2	2	0	1	4	3	1	1	0
4. Aperture width : shell width	2	2	4	1	4	4	3	3	0
5. Shell height: shell length	2	2	2	2	4	4	1	2	0
6. Shell thickness 1 : shell length	0	0	0	0	2	4	0	0	4
7. Shell thickness 2 : shell length	1	3	2	1	1	3	2	0	4
8. Body weight : shell length	4	4	4	1	2	4	3	1	0
9. Shell weight : shell length	3	4	2	1	1	2	1	0	0
10. Operculum length : shell length	4	4	3	1	4	4	4	0	?
11. Operculum width : shell width	4	1	3	2	3	2	4	0	?
12. Operculum width : operculum lengt	2	0	2	4	4	4	1	3	?
13. Aperture width : aperture length	2	3	4	3	3	4	3	3	0
14. Shell length	4	4	3	2	2	3	3	1	0
15. Rib strength	?	1	2	2	1	0	1	0	2
16. Number of ribs on body whorl	1	1	2	2	1	0	3	0	2
17. Aperture colour	0	0	0	0	1	1	0	1	0
18. Presence of axial ribs on spire	0	0	0	1	0	0	1	0	1
19. Shell covered with bryozoan	0	0	1	1	0	0	1	0	0
20. Constriction	4	3	0	2	4	0	2	3	2
21. Aperture sculpture	0	4	3	3	1	4	4	1	0
22. Parietal scar	2	3	2	2	4	4	2	4	2
23. Spire angle	3	3	3	2	3	4	1	1	0

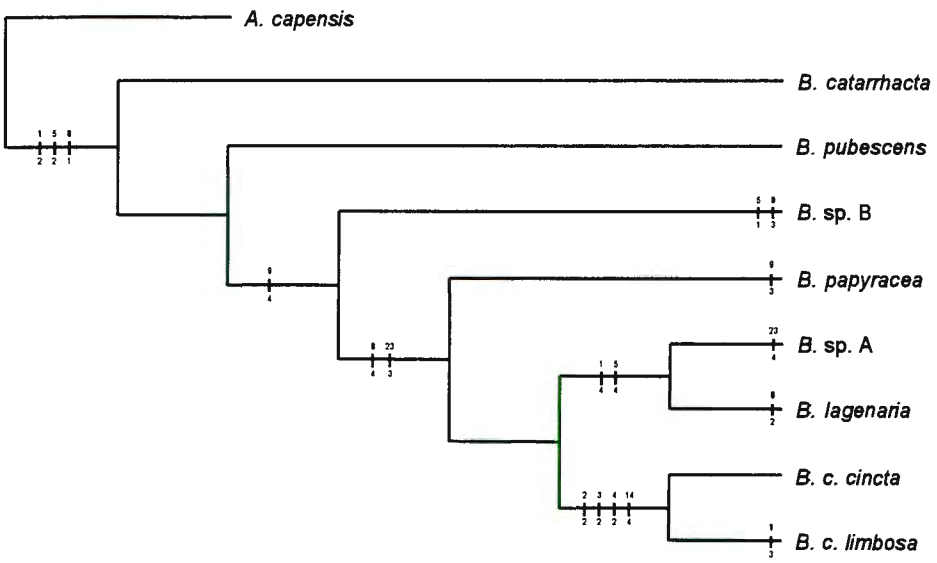

Figure 5. Cladogram illustrating the relationships among the species of *Burnupena* based on the morphological data. The bars at nodes represent synapomorphies, whilst those at the terminals indicate autapomorphies for characters shown elsewhere on the tree (otherwise autapomorphies are not shown). The numbers indicate the character (above) and its state (below),

23), two were treated as unordered multistate characters (15 and 16), and three were binary characters (17 to 19). A single, fully resolved, most parsimonious cladogram was found (Fig. 5), with a length of 132, a consistency index of 0.59 and a retention index of 0.48. As with the cladograms constructed from the allozyme data, *B. catarrhacta* is placed as the sister species to all other *Burnupena* species, although, unlike the allozyme cladograms which are very well supported, this is not supported by any synapomorphies. The two *B. cincta* subspecies are shown as sister species, and, unlike the allozyme cladograms, is well supported by several synapomorphies. These two species together with *B. lagenaria* and *B.* sp. A form a monophyletic group. The topology of this tree is very similar to that of the Wagner tree (Fig. 1), the only difference being the position of *B.* sp. A, shown clustering with *B. lagenaria* in the morphological cladogram, but joined to *B.* sp. B in the Wagner tree. The main differences between the allozyme and morphological cladograms (Figs. 2 to 5) are the morphological placement of *B.* sp. A as a relatively recent species, and the positioning of *B. pubescens* outside of the cluster containing *B. papyracea*.

DISCUSSION

As noted in the introduction, cladistic analyses are critically dependent on the choice of the outgroup taxa which determines the polarity of the characters. In all of the analyses, *B. catarrhacta* was shown to be the sister species to all other *Burnupena* species (which formed a monophlyetic group), and can therefore be regarded as more basal. Swofford and Olsen (1990) emphasized that the assignment of a taxon to the outgroup constitutes an assumption that the remaining ingroup taxa are monophyletic. To test the choice of *A. capensis* as the outgroup taxon, all analyses were repeated using *B. catarrhacta* as outgroup to root the trees. In no case was the topology of the tree altered as a consequence.

One of the problems associated with using phenograms to estimate phylogenies is that many of the methods require the assumption of equal rates of divergence. Different branch lengths may reflect either different rates of divergence or length of time since separation. As noted, the long branch length of *B. catarrhacta* in the Wagner tree (Fig. 1) is more likely to be due to a longer time since separation for two reasons. Firstly, the distance of all of the species to the root is similar, and secondly, the topology of a dendrogram produced using the UPGMA clustering method, which does assume equal rates of divergence, was identical to the Wagner tree. Further, midpoint rooting also requires the same assumption of equal rates of divergence among lineages (Hillis, 1987), and in this study midpoint rooting produced a Wagner tree with the same topology, due to the large genetic distance between *B. catarrhacta* and the other species. This is not always the case, however. In a genetic study of lizards belonging to the genus *Pholidobolus*, Hillis (1985) found that, although distance procedures provided a good approximation of the most parsimonious cladogram, the use of midpoint rooting destroyed most of the phylogenetic information. He also suggested that, due to unequal rates of divergence, a molecular clock based on electrophoretic data should not be an *a priori* assumption in electrophoretic studies. The topology of the Wagner tree constructed from genetic distances (Fig. 1) was not unlike the cladograms produced from the allozyme data (Figs. 2 - 4), except for the placement of *B. pubescens*. Apart from *B. catarrhacta*, this species had more than

twice as many species-specific alleles (autapomorphs) than the other species. This would probably account for its placement in the Wagner tree as a more distantly related species, since the genetic distance would reflect these unique alleles. The presence of autapomorphs, however, are not reflected in the cladograms since they are not used.

The consistency indices obtained for the cladograms based on both the allozyme and morphometric data sets were similar, ranging from 0.55 to 0.65, indicating that approximately 40% of the character state changes could be ascribed to homoplasy. These values are within the range reported for other molluscan studies (CI = 0.43 for allozyme data and 0.70 for morphometric data for 13 species of the freshwater mussel *Anodonta*, Hoeh, 1990; CI = 0.67 based on morphological characters for all species of *Littorina*, Reid, 1990; CI = 0.71 based on combined allozyme and morphological characters for representatives of 23 genera of polygyrid land snails, Emberton, 1994b; CI = 0.36 based on allozyme data for 18 species of acavid land snails, Emberton, 1995; CI = 0.76 based on allozyme data for 11 species of *Biomphalaria*, a fresh water snail, Bandoni et *al*., 1995).

All three of the cladograms produced from the allozyme data are consistent with each other, differing only in the degree of resolution (Figs. 2 - 4). This is to be expected since the same data set was employed, differing only in the methods of coding the data. Thus it would appear that for this data set, the choice of coding method is not too critical. Amongst four recent studies of phylogeny of molluscs, two, Hoeh (1990) and Emberton (1995) used the allele as the character, with the other two, Emberton (1994b) and Bandoni et *al*. (1995) using the locus as the character. In the former, the *minimum allele turnover* model was employed to order the states, but in the latter, the states were left unordered. In this study, a separate analysis with the states unordered, produced 13 equally parsimonious trees (L=101, CI=0.94, RI=0.57), and the consensus tree was completely unresolved. For these allozyme data, it is clear that ordering the states increased the phylogenetic informativeness of the characters.

The results of the allozyme cladograms (Figs. 2 - 4) all support the conclusion that the genus is monophyletic, and that *B. catarrhacta* is the most basal species, and is a sister species to all of the other species of *Burnupena*, which form a monophyletic clade. The cladograms also show that *B. lagenaria* and the two subspecies of *B. cincta* constitute a distinct and closely related cluster, which is also supported by the morphometric data, and confirms the results of the multivariate analyses and population genetic studies from the first two Chapters. *B. c. cincta* and *B. c. limbosa* are shown as closely related taxa (Fig. 3), which supports their subspecific status. That *B. papyracea* and *B. pubescens* are closely related is also supported by all three cladograms, as is the monophyly of *B. papyracea*, *B. pubescens*, *B. c. cincta*, *B. c. limbosa* and *B. lagenaria*, indicating that they all share the same common ancestor.

The cladogram produced from the morphological data (Fig. 5) was generally more similar to the Wagner tree (Fig. 1) than to the allozyme cladograms (Fig. 2 - 4). It does differ from all of these in the placement of *B.* sp. A. The apparently close relationship between *B. lagenaria* and *B.* sp. A would appear to reflect the overall similarity in shell shape between these two species, as detected in the multivariate analyses discussed in Chapter 1, leading to the conclusion that this relationship may be questionable. As noted above, quantitative data are not considered to be useful in reconstructing phylogenies (Pimental & Riggins, 1987; Cranston & Humphries, 1988; Chappill, 1989). However, a comparison of cladograms from the morphometric and allozyme data sets does indicate a striking number of similarities. Since these are independent data sets, congruencies between them would lend support for particular clades. In particular, there is support for the close relationship between *B. c. cincta* and *B. c. limbosa*, and the close relationship of *B. lagenaria* with these two species. The placement of *B. catarrhacta* as the sister to the other species is also strongly supported. Only the placement of *B.* sp. A with *B. lagenaria* is not supported.

The hypothesised phylogeny based on the allozyme data is considered to be a more accurate reflection of the true phylogeny of *Burnupena*. The placement of *B.* sp. A and *B.* sp. B is, however, not clear. Due to the small sample size of *B.* sp. B (19), its position within the cladogram should be regarded with caution, since the possibility exists that alleles present in the population at

low frequencies may not have been detected, and if possession of such an allele defines a clade, then this species could become excluded from that clade. Swofford and Berlocher (1987) produced a table showing the probability of failing to detect low-frequency alleles in samples of various sizes: for example, an allele occurring at a frequency of 0.05 will be missed in about 13% of all 20-individual samples (and about 5% for sample sizes of 30). All the other species (populations pooled) have sample sizes of 47 - 231. Furthermore, the placement of *B.* sp. A as the sister to *B.* sp. B (Fig. 2) is only supported by a single synapomorphy.

Overall, the most clear results emerging are the position of *B. catarrhacta* as the most basal species, and the monophyly of *B. papyracea, B. pubescens, B. c. cincta, B. c. limbosa* and *B. lagenaria*, both of which are well supported.

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Appendix A. Allele frequencies for the polymorphic loci in the eight *Burnupena* species and *A. capensis*. The maximum number of individuals examined is shown in parentheses. An * indicates that the allele is present in both *Burnupena* and *A. capensis*. Two of the loci found *Burnupena* (*AAT-2* and *ME-1*) could not be scored (ns) for *A. capensis*. Species abbreviations are as in Table 1.

	Species								
Locus/ allele	CIN (231)	LIM (106)	PAP (194)	PUB (78)	LAG (164)	A (68)	B (19)	CAT (47)	CAP (28)
ARK									
2	-	-	-	-	-	-	-	-	0.25
32*	-	-	-	0.045	-	-	-	-	0.61
52*	0.756	0.656	0.082	0.455	0.936	0.985	0.316	-	0.14
70	0.244	0.344	0.013	0.500	0.064	0.007	0.684	-	-
82	-	-	-	-	-	-	-	1.000	-
86	-	-	0.407	-	-	-	-	-	-
100	-	-	0.497	-	-	0.007	-	-	-
AAT-1									
-100	1.000	1.000	0.997	0.987	1.000	1.000	0.947	-	-
-70	-	-	-	-	-	-	-	-	0.02
-65	-	-	0.003	0.013	-	-	-	-	-
-61	-	-	-	-	-	-	0.053	-	-
-50	-	-	-	-	-	-	-	1.000	-
-45	-	-	-	-	-	-	-	-	0.98
AAT-2									
65	0.135	0.148	0.137	0.014	0.024	0.493	1.000	-	ns
100	0.865	0.852	0.863	0.986	0.976	0.507	-	0.660	
127	-	-	-	-	-	-	-	0.340	
DIA-1									
91	-	-	-	-	-	-	-	-	1.000
94	-	-	0.372	1.000	0.095	1.000	0.974	1.000	-
100	1.000	1.000	0.628	-	0.905	-	0.026	-	-
G6PDH									
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000
104	-	-	-	-	-	-	-	1.000	-
GPI									
-165	-	-	-	0.006	-	-	-	-	-
-150	-	-	-	-	-	-	-	1.000	-
-86	-	-	0.003	-	-	-	-	-	-
100	0.991	1.000	0.992	0.212	1.000	1.000	0.974	-	-
180	-	-	-	-	-	-	-	-	0.16
200	-	-	-	0.173	-	-	-	-	-
315	0.009	-	0.005	0.442	-	-	0.026	-	-
365	-	-	-	-	-	-	-	-	0.73
450	-	-	-	0.160	-	-	-	-	-
490	-	-	-	-	-	-	-	-	0.11
575	-	-	-	0.006	-	-	-	-	-
GAP									
-520	-	-	-	-	-	-	-	0.598	-
-450	-	-	-	-	-	-	-	0.402	-
-420	-	-	-	-	-	-	-	-	0.92
-100*	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	0.08
HEX-3									
92	-	-	-	-	0.046	-	-	-	-
95	-	-	0.003	0.026	-	-	-	-	-
97	-	-	-	-	-	-	-	0.553	-
98	-	-	0.021	-	0.003	0.007	-	-	-
100	1.000	1.000	0.977	0.974	0.951	0.993	1.000	-	-
101	-	-	-	-	-	-	-	0.447	-
103	-	-	-	-	-	-	-	-	1.000

(cont.)

Appendix A (continued)

Locus/ allele	Species								
	CIN	LIM	PAP	PUB	LAG	A	B	CAT	CAP
IDH-1									
10	0.004	-	0.003	-	-	-	-	-	-
100	0.885	0.995	0.949	0.993	0.963	0.194	1.000	1.000	-
200	-	-	-	-	-	-	-	-	0.73
236	0.111	0.005	0.048	0.007	0.037	0.806	-	-	-
290	-	-	-	-	-	-	-	-	0.27
IDH-2									
83	0.002	-	-	0.019	0.061	-	0.026	-	-
100	0.998	1.000	1.000	0.974	0.933	1.000	0.974	1.000	-
117	-	-	-	0.006	0.006	-	-	-	-
130	-	-	-	-	-	-	-	-	1.000
LAP									
90	-	-	-	-	-	-	-	1.000	-
100	0.578	0.198	1.000	0.051	0.003	-	-	-	-
103	-	-	-	-	-	1.000	1.000	-	-
106*	0.413	0.651	-	0.679	0.040	-	-	-	0.12
109*	0.009	0.151	-	0.269	0.957	-	-	-	0.88
LDH									
99	-	-	-	-	-	-	-	-	1.000
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-
MDH-1									
-280	-	-	-	-	-	-	-	1.000	-
-65	-	-	-	-	-	-	0.237	-	-
-40	-	-	-	-	-	-	-	-	0.08
-32	-	-	0.034	0.831	-	-	-	-	-
80	-	-	-	-	-	-	-	-	0.92
100	1.000	1.000	0.966	0.169	1.000	1.000	0.763	-	-
MDH-2									
-67	-	-	-	-	-	-	-	1.000	-
36	-	-	-	1.000	-	-	-	-	-
100	1.000	1.000	1.000	-	1.000	1.000	1.000	-	-
103	-	-	-	-	-	-	-	-	1.000
MDH-3									
76	-	-	-	-	-	-	-	-	1.000
82	-	0.005	-	-	-	-	-	-	-
95	-	-	-	-	-	-	-	1.000	-
100	1.000	0.995	1.000	1.000	1.000	1.000	1.000	-	-
ME-1									
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	ns
ME-3									
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-	1.000
102	-	-	-	-	-	-	-	1.000	-
MPI									
75	-	0.005	-	0.667	0.015	-	-	-	-
78	-	-	-	-	-	-	-	1.000	-
88*	0.333	0.236	-	-	-	0.941	0.395	-	0.04
97*	0.550	0.623	-	0.333	0.963	0.059	-	-	0.06
100*	-	-	0.992	-	0.006	-	0.474	-	0.09
106*	0.117	0.137	0.008	-	0.015	-	0.132	-	0.81

(cont.)

Appendix A (continued)

Locus/ allele	Species CIN	LIM	PAP	PUB	LAG	A	B	CAT	CAP
ODH									
62	0.165	0.073	-	-	-	-	0.053	-	-
69	-	-	0.008	-	-	-	-	-	-
82	0.325	0.536	-	-	0.699	-	-	-	-
83	-	-	-	-	-	-	-	-	0.02
85	-	-	-	0.058	-	-	-	-	-
100	0.509	0.380	0.859	0.935	0.301	1.000	0.947	-	-
115*	-	0.010	0.132	0.006	-	-	-	-	0.13
125	-	-	-	-	-	-	-	1.000	-
130	-	-	-	-	-	-	-	-	0.85
GL									
80	-	-	-	-	-	-	-	-	0.09
91	-	-	0.003	-	-	-	-	-	-
100*	0.065	0.241	0.526	-	0.133	0.079	0.026	-	0.24
105	-	-	-	-	-	-	-	-	0.67
111	-	-	0.472	1.000	-	0.794	0.974	-	-
115	0.900	0.759	-	-	0.867	0.127	-	-	-
119	0.035	-	-	-	-	-	-	-	-
129	-	-	-	-	-	-	-	1.000	-
PHP									
70	-	-	-	-	-	-	-	-	0.34
78	-	-	-	-	-	-	-	-	0.66
81	-	-	-	-	-	0.007	-	-	-
87	-	-	-	-	-	0.110	-	-	-
95	-	-	-	-	-	-	-	1.000	-
100	1.000	1.000	1.000	0.974	0.979	0.882	1.000	-	-
104	-	-	-	0.026	0.021	-	-	-	-
PGM									
69	0.146	0.132	0.003	-	0.201	-	-	0.553	-
86	0.352	0.382	0.003	-	0.238	-	-	0.447	-
91	-	-	-	-	-	-	-	-	0.73
100*	0.471	0.472	0.832	-	0.561	0.934	0.947	-	0.07
102	-	-	-	-	-	-	-	-	0.20
111	0.031	0.014	0.149	0.218	-	0.066	0.053	-	-
121	-	-	0.013	0.782	-	-	-	-	-
PGD									
-800	-	-	-	-	-	-	-	-	0.32
-655	-	-	-	-	-	-	-	1.000	-
-490	-	-	-	-	0.806	-	-	-	-
-455*	-	-	0.997	-	-	0.044	-	-	0.68
-300	-	-	-	-	-	0.956	-	-	-
-260	-	-	-	-	-	-	0.158	-	-
-182	-	-	-	0.662	-	-	0.026	-	-
-100	1.000	1.000	0.003	0.020	0.194	-	0.816	-	-
455	-	-	-	0.311	-	-	-	-	-
800	-	-	-	0.007	-	-	-	-	-
SDH									
77	-	-	-	-	-	-	-	1.000	-
86	-	-	-	-	-	-	-	-	1.000
100	0.865	0.967	0.960	0.799	0.985	1.000	1.000	-	-
106	0.135	0.033	0.040	0.174	0.015	-	-	-	-
118	-	-	-	0.028	-	-	-	-	-
SOD									
100	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	-
110	-	-	-	-	-	-	-	-	1.000

Appendix B. Character step matrices of observed allele combinations (states) for all characte (loci), with pairwise distances between states. Alleles were labelled alphabetically to make a combinations easier to read. Each unique combination was coded as a separate state. All matrices are symmetrical.

Locus/ allele	allele label	states	code	step matrices

ARK

	0	1	2	3	4	5
0	-	3	5	2	4	4
1	3	-	2	1	1	3
2	5	2	-	3	1	5
3	2	1	3	-	2	4
4	4	1	1	2	-	4
5	4	3	5	4	4	-

Locus/ allele	allele label	states	code
2	a	abc	0
32	b	cd	1
52	c	cdfg	2
70	d	bcd	3
82	e	cdg	4
86	f	e	5
100	g		

AAT-1

	0	1	2	3	4
0	-	2	3	3	2
1	2	-	1	1	2
2	3	1	-	2	3
3	3	1	2	-	3
4	2	2	3	3	-

Locus/ allele	allele label	states	code
-100	a	bf	0
-70	b	a	1
-65	c	ac	2
-61	d	ad	3
-50	e	e	4
-45	f		

AAT-2

	0	1	2
0	-	1	2
1	1	-	3
2	2	3	-

Locus/ allele	allele label	states	code
65	a	ab	0
100	b	a	1
127	c	bc	2

DIA-1

	0	1	2	3
0	-	2	3	2
1	2	-	1	2
2	3	1	-	1
3	2	2	1	-

Locus/ allele	allele label	states	code
91	a	a	0
94	b	c	1
100	c	bc	2
		b	3

GPI

	0	1	2	3	4	5
0	-	5	4	6	8	4
1	5	-	1	1	4	3
2	4	1	-	2	5	2
3	6	1	2	-	5	4
4	8	4	5	5	-	7
5	4	3	2	4	7	-

Locus/ allele	allele label	states	code
-165	a		
-150	b		
-86	c		
100	d	ehj	0
180	e	dg	1
200	f	d	2
315	g	cdg	3
365	h	adfgik	4
450	i	b	5
490	j		
575	k		

GAP

	0	1	2
0	-	1	4
1	1	-	3
2	4	3	-

Locus/ allele	allele label	states	code
-520	a	cd	0
-450	b	d	1
-420	c	ab	2
-100	d		

HEX-3

	0	1	2	3	4	5	6
0	-	2	4	3	4	3	3
1	2	-	2	1	2	1	3
2	4	2	-	1	2	1	5
3	3	1	1	-	3	2	4
4	4	2	2	3	-	1	5
5	3	1	1	2	1	-	4
6	3	3	5	4	5	4	-

Locus/ allele	allele label	states	code
92	a	g	0
95	b	e	1
97	c	bde	2
98	d	be	3
100	e	ade	4
101	f	de	5
103	g	cf	6

(cont.)

Appendix B (continued)

Locus/ allele	allele label	states	code	step matrices								
IDH-1				0	1	2	3					
10	a	ce	0	-	5	4	3					
100	b	abd	1	5	-	1	2					
200	c	bd	2	4	1	-	1					
236	d	b	3	3	2	1	-					
290	e											
IDH-2				0	1	2	3					
83	a	d	0	-	4	2	3					
100	b	abc	1	4	-	2	1					
117	c	b	2	2	2	-	1					
130	d	ab	3	3	1	1	-					
LAP				0	1	2	3	4				
90	a	de	0	-	1	3	3	3				
100	b	bde	1	1	-	2	4	4				
103	c	b	2	3	2	-	2	2				
106	d	c	3	3	4	2	-	2				
109	e	a	4	3	4	2	2	-				
LDH				0	1							
99	a	a	0	-	2							
100	b	b	1	2	-							
MDH-1				0	1	2	3	4				
-280	a	ce	0	-	2	3	3	2				
-65	b	f	1	2	-	1	1	2				
-40	c	df	2	3	1	-	2	3				
-32	d	bf	3	3	1	2	-	3				
80	e	a	4	2	2	3	3	-				
100	f											
MDH-2				0	1	2	3					
-67	a	d	0	-	2	2	2					
36	b	c	1	2	-	2	2					
100	c	b	2	2	2	-	2					
103	d	a	3	2	2	2	-					
MDH-3				0	1	2	3					
76	a	a	0	-	2	3	2					
82	b	d	1	2	-	1	2					
95	c	bd	2	3	1	-	3					
100	d	c	3	2	2	3	-					
MPI				0	1	2	3	4	5	6	7	8
75	a	cdef	0	-	1	2	2	4	2	2	1	5
78	b	cdf	1	1	-	1	3	3	3	1	2	4
88	c	acdf	2	2	1	-	4	2	2	2	3	5
97	d	ef	3	2	3	4	-	4	2	4	1	3
100	e	ad	4	4	3	2	4	-	2	2	5	3
106	f	adef	5	2	3	2	2	2	-	4	3	5
		cd	6	2	1	2	4	2	4	-	3	3
		cef	7	1	2	3	1	5	3	3	-	4
		b	8	5	4	5	3	3	5	3	4	-

(cont.)

Appendix B (continued)

Locus/ allele	allele label	states	code	step matrices

ODH

	0	1	2	3	4	5	6	7	8
62 a dgi 0	-	6	7	6	6	5	4	5	4
69 b acf 1	6	-	1	4	4	1	2	1	4
82 c acfg 2	7	1	-	3	3	2	3	2	5
83 d bfg 3	6	4	3	-	2	3	2	3	4
85 e efg 4	6	4	3	2	-	3	2	3	4
100 f cf 5	5	1	2	3	3	-	1	2	3
115 g f 6	4	2	3	2	2	1	-	1	2
125 h af 7	5	1	2	3	3	2	1	-	3
130 i h 8	4	4	5	4	4	3	2	3	-

GL

	0	1	2	3	4	5	6	7
80 a acd 0	-	4	3	4	4	4	3	4
91 b cfg 1	4	-	1	4	4	2	3	4
100 c cf 2	3	1	-	3	3	1	2	3
105 d bce 3	4	4	3	-	2	2	1	4
111 e e 4	4	4	3	2	-	2	1	2
115 f cef 5	4	2	1	2	2	-	1	4
119 g ce 6	3	3	2	1	1	1	-	3
129 h h 7	4	4	3	4	2	4	3	-

PHP

				0	1	2	3	4
70 a								
78 b	ab	0		-	3	4	5	3
81 c	f	1		3	-	1	2	2
87 d	fg	2		4	1	-	3	3
95 e	cdf	3		5	2	3	-	4
100 f	e	4		3	2	3	4	-
104 g								

PGM

			0	1	2	3	4	5	6
69 a	cde	0	-	5	6	5	4	3	5
86 b	abdf	1	5	-	1	4	1	2	2
91 c	abdfg	2	6	1	-	3	2	3	3
100 d	fg	3	5	4	3	-	5	2	4
102 e	abd	4	4	1	2	5	-	3	1
111 f	df	5	3	2	3	2	3	-	4
121 g	ab	6	5	2	3	4	1	4	-

PGD

			0	1	2	3	4	5	6	7
-800 a										
-655 b	ad	0	-	3	2	6	4	2	5	3
-490 c	h	1	3	-	1	3	1	3	2	2
-455 d	dh	2	2	1	-	4	2	2	3	3
-300 e	ghij	3	6	3	4	-	4	6	3	5
-260 f	ch	4	4	1	2	4	-	4	3	3
-182 g	de	5	2	3	2	6	4	-	5	3
-100 h	fgh	6	5	2	3	3	3	5	-	4
455 i	b	7	3	2	3	5	3	3	4	-
800 j										

SDH

			0	1	2	3	4
77 a	b	0	-	3	4	2	2
86 b	cd	1	3	-	1	1	3
100 c	cde	2	4	1	-	2	4
106 d	c	3	2	1	2	-	2
118 e	a	4	2	3	4	2	-

SOD

			0	1
100 a	b	0	-	2
110 b	a	1	2	-

Chapter 6

Detection of two coexisting species of Oxystele (Gastropoda: Trochidae) by morphological and electrophoretic analyses

INTRODUCTION

Oxystele variegata (Anton, 1838) is a very common intertidal topshell which is distributed around most of the Southern African coastline and occurs at mid-tide level (in the balanoid zone)(Day, 1969; Kilburn & Rippey, 1982). It is abundant to such an extent that it has been listed as one of the two species with the highest biomass on certain rocky intertidal shores in the Cape Peninsula (McQuaid & Branch, 1984; McQuaid, Branch & Crowe, 1985). In one of the pioneering experiments on the relationship between zonation and stress-tolerance in gastropods, Broekhuysen (1941) showed that it is very tolerant of temperature and salinity stress, as befits its mid- to high-shore zonation. McQuaid (1982, 1983) described *O. variegata* as exhibiting a vertical size gradient, with size increasing in an upshore direction. He carried out experiments on its vertical migration, desiccation resistance and susceptibility to predation, and concluded that *O. variegata* undergoes vertical migration on an age dependent basis, and that its zonation is determined by an interplay between predation and desiccation.

The feature generally considered to be distinctive of *O. variegata* is a necklace of small, dark and light squares immediately below the suture (Barnard, 1963; Richards, 1981; Kilburn & Rippey, 1982). However, initial examination of *O. variegata* snails in the Cape Peninsula revealed several colour differences between some of the shells, raising doubts whether only one single species was indeed at hand. Further studies, including scanning electron microscopy and enzyme electrophoresis, revealed structural and enzymic differences between the two forms. We show that the two forms are consistently different in their shell patterns, radulae and biochemistry, leading us to believe that they are actually two different species, *impervia* and *variegata*, which we hereby describe. All of the earlier work described above for "*O. variegata* " is likely to have been based on both species.

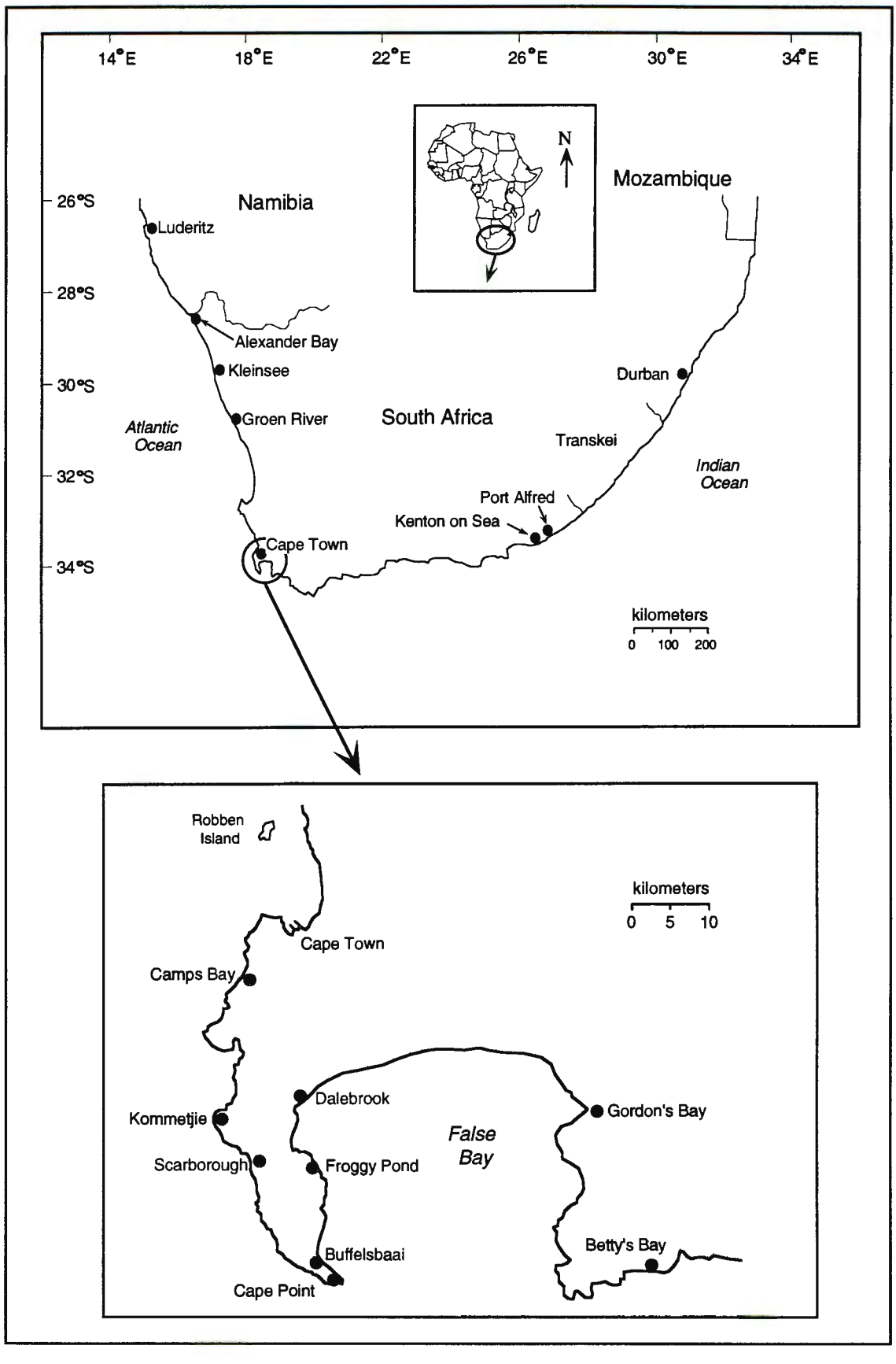


Figure 1. Map showing localities at which *O. impervia* and *O. variegata* were sampled. The range of '*O. variegata*' as indicated by Day (1969) is also shown. Inset: Sampling localities in and around the Cape Peninsula.

METHODS

MATERIAL

Animals were collected from nine sites around the Cape Peninsula, as well as from three sites on the West Coast and two on the South-eastern Cape coast (Fig. 1), from February to April, 1989. These sites were: Alexander Bay, Brazil (near Kleinsee) and Groen River, on the West Coast; Camps Bay, Kommetjie, Scarborough, Cape Point, Buffelsbaai, Froggy Pond, Dalebrook, Gordon's Bay and Betty's Bay, all in and around the Cape Peninsula; and Kenton on Sea and Port Alfred, both in the South-eastern Cape. At most of these sites, the two presumed species occurred sympatrically. Specimens from 10 of the sites (excluding Alexander Bay, Cape Point, Gordon's Bay and Betty's Bay) were used for radular and electrophoretic studies. Altogether, 1056 snails were collected. Of these, 633 were used to study shell colour variation. These shells have since been deposited in the South African Museum (Catalogue numbers SAM-A37548 to SAM-A37561), with voucher specimens in the Natal Museum in Pietermaritzburg. The remaining 423 served first to study shell variation, and then were used for radular and electrophoretic studies. Five specimens of each of the three other South African *Oxystele* species were collected for comparison (*O. sinensis* (Gmelin, 1791) from Kenton on Sea, *O. tabularis* (Krauss, 1848) from Port Alfred, and *O. tigrina* (Anton, 1839) from Scarborough).

RADULAE

Using scanning electron microscopy, the radulae of up to nine specimens of each putative species were examined for each of the 10 sites. All radulae were taken from the animals used for electrophoresis. The radulae were first cleaned by shaking for 10 minutes in a 10% solution of potassium hydroxide, then transferred to 70% alcohol and cleaned for 30 seconds in an ultrasonic bath. Finally, they were mounted on stubs, gold-palladium coated and examined in a Cambridge

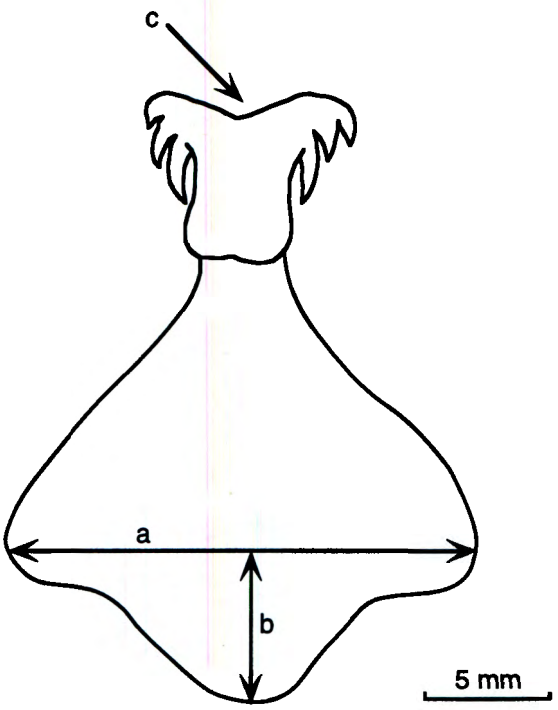


Figure 2. A diagramatic representation of the central radular tooth to show: (a) the broadest width; (b) the perpendicular to the basis of the tooth; (c) the indentation of the cusp.

S-200 Electron Microscope. For each radula, one typical central tooth was chosen and photographed. From this photograph, two measurements were taken: a) the broadest width of the tooth; b) a perpendicular bisecting this line, and dropping to the base of the tooth (Fig. 2). The ratio between these two measurements (b:a) was then calculated (and is termed the "basal ratio"). The data from all the sites were pooled for each of the two putative species, and a Mann-Whitney test performed to test for significant differences.

A further radular feature examined by SEM was the extent of indentation of the cusp of the central tooth (Fig. 2). Initially, we quantified this character by classifying the cusp into six "indentation categories" from (1) slight, to (6) deeply indented. Later, for the taxonomic analysis (which requires clear-cut diagnostic characters) we lumped the first three categories into a "slightly indented" group, and the three last into a "well indented" one. Altogether we measured cusp indentation on 114 individuals (53 *impervia* and 61 *variegata*). Statistical analysis of the indentation differences between the two species was performed by use of contingency table analysis, using the log-likelihood ratio test (G-test).

ELECTROPHORESIS

Samples were prepared on the day of collection as far as possible, but those from the West Coast and South-eastern Cape were kept in circulating sea water for a few days. Preliminary analysis showed that results from samples kept in the aquarium for longer than about a week, or those frozen whole at -20°C overnight, were not as good as those from freshly prepared tissues. Samples from each site were first separated into the two presumed species, giving a total of 18 populations. (No *impervia* samples were collected at two of the sites. At Brazil both species were found in a preliminary sample but, by the time they reached us, they were not fresh enough to use for electrophoresis. Only *variegata* was found in a second sample, collected for us a month later. At Buffelsbaai, only one *impervia* individual was found.)

Table 1. Enzymes, locus abbreviations and buffers used in this study.

Enzyme (EC number)	Locus	Buffer *
Adenylate kinase (2.7.4.3)	*Ak*	1
Arginine kinase (2.7.3.3)	*Ark*	2
Glucosephosphate isomerase (5.3.1.9)	*Gpi*	2
Leucine aminopeptidase (3.4.11.-)	*Lap*	1
Peptidase: Glycyl-leucine (3.4.11.-)	*Gl*	2
6-Phosphogluconate dehydrogenase (1.1.1.44)	*Pgd*	1

* 1 = electrode (pH 8.0) LiOH 0.06M, boric acid 0.3M; gel (pH 8.7) tris 0.03M, citric acid 0.005M, LiOH 0.006M, boric acid 0.03M (Ridgway et al., 1970).
2 = electrode (pH 6.1) citric acid 0.04M, n-(3-aminopropyl) morpholine 10.5ml/l; gel (pH 6.1) 1:19 dilution of electrode buffer plus 1 drop morpholine per 100ml gel buffer (Clayton & Tretiak, 1972).

The visceral mass and radula were removed from each animal and the remaining tissue homogenised in 0.01M Tris buffer (pH 8.0). Samples were frozen overnight at -20°C and then on the following day, thawed and centrifuged for ten minutes prior to electrophoresis. Horizontal starch gel electrophoresis followed the method of May *et al.* (1979), and staining methods were based on recipes in Harris & Hopkinson (1976) and Shaw & Prasad (1970).

Initially, in the preliminary stages of the study, 22 enzymes were examined. Of these, six enzymes were chosen which gave clear repeatable results, were polymorphic, and showed differences between the two suspected species (Table 1). Two buffer systems were used (Table 1). Alleles were designated by their mobilities relative to the most common allele (designated 100) in the Dalebrook population which was used as the reference population. Alleles which migrated cathodically were indicated by a minus sign.

Deviations from Hardy-Weinberg proportions were tested for each locus using the G-test for goodness of fit (Sokal & Rohlf, 1969). Contingency table analysis was used to test for significant genetic differentation among populations. When multiple tests of the same hypothesis are made, the chance of making a type 1 error is increased (Cooper, 1968). To compensate for this, the probability level of the rejection criteria was adjusted by dividing the alpha value chosen by the number of tests made. Nei's (1978) unbiased genetic identity, I, and genetic distance, D, were calculated, and a cluster analysis performed on the distance matrix, using Sneath and Sokal's (1973) unweighted pair-group method with arithmetic averaging (UPGMA).

RESULTS

MORPHOLOGICAL DIAGNOSIS OF THE TWO SPECIES

Oxystele impervia (Menke, 1843)

Plate 1 (a-l)

Turbo impervius Menke, 1843: 18.

Trochus impervius (Menke) Philippi, 1844-1846. pl. 4 (fig. 5).

Trochus impervius (Menke) Philippi, 1851. pl. 24 (fig. 8).

Oxystele sagittifera perdix (Koch) Turton, 1932: pl. 44 (no. 1280).

Oxystele carinata Turton, 1932: pl. 44 (no. 1282).

Oxystele variegata (Anton 1839) Richards, 1981: 67, 67a.

Oxystele variegata (Anton) Branch & Branch, 1981: 115-d.

Oxystele variegata Kilburn & Rippey, 1982: pl. 9-4 (two right shells).

Oxystele variegata Kensley & Penrith, 1972: pl. 13 (a - upper row; b - mid row, rightmost).

Neotype No. SAM-A37548, from Dalebrook, Cape Peninsula, in the South African Museum, Cape Town (Plate 1a-b).

Diagnosis: Shell of medium size (shell diameter up to 27 mm, shell height up to 23 mm) with a texture that is usually smooth. The pattern of the shell consists of two elements, ground colour and spiral bands.

The ground colour consists of shades of yellow (c,d), brown (i-l), orange (f) or red (g,h). In a single shell, these colours may change gradually or abruptly (j), both in hue (e.g. from pink to yellow (e)) and in tone (j)(e.g. from beige through greyish- yellow to brownish-yellow, yellowish-brown, light brown, brown or dark brown).

Plate 1. Shells of *O. impervia* (a-l) and *O. variegata* (m-x). *O. impervia*: (a, b) neotype, Dalebrook; (c, f) Groen River; (d, i, l) Kleinsee; (e, g, h, j, k) Cape Point. *O. variegata*: (m, n, o) neotype, Dalebrook; (p) 'neoparatype', Dalebrook; (q) Kenton on Sea; (r, s, w) Groen River; (t, u) Betty's Bay; (v) Dalebrook; (x) Kleinsee.

The spiral bands consist of alternating dark and off-white maculations. The dark maculations are usually of a hue that is similar to that of the ground colour (red (g), orange, brown (a,j), yellow (d)) but they are usually of a slightly darker shade. The uppermost band on each whorl (which we call the "necklace"), near the suture, is the most prominent one, both in its breadth and in its darkness (d,g,k); its pale maculations are similar to the ground colour, or slightly paler.

In some shells there are no, or only very faint, spiral bands between the necklace and the crest of the whorl (c,d,i). Sometimes all the spiral bands are of a similar colour (a,c) but the 2-3 bands of the crest of the whorl may be darker than the rest, their dark dashes resembling the colour of the necklace (d,f,g,k).

The radula of *O. impervia* (Plate 2a) differs from that of *O. tigrina*, (Plate 2b) in that the central and lateral teeth are not as elongate. The base of the central tooth is usually shallow (Plate 2c), so that the basal ratio is small (Fig. 3a). The cusp of the central tooth is almost always only slightly indented (88% in our samples;Table 2). In addition to the two-category grouping of indentation, Table 2 also presents our original six-category classification, so as to give a somewhat more detailed picture of the extent of intraspecific variation. It shows that well-indented cusps are uncommon in *O. impervia*, and that the two highest indentation categories comprise only 4% of the samples.

There was no suggestion of a geographic cline in the mean basal ratio of the radula. However, we had very few specimens per site, so this conclusion is tentative.

Distribution and habitats: Our most northern samples of *O. impervia* come from Kleinsee (550 km north of Cape Town) on the West Coast; our most eastern ones, from Port Alfred (1000 km east of Cape Town) on the South-eastern Cape coast. Subsequently, this species has also been recorded in low numbers at Dwesa (in Transkei), some 240 km east of Port Alfred (pers. comm.). *O. impervia* thus spans a range of at least 1740 km.

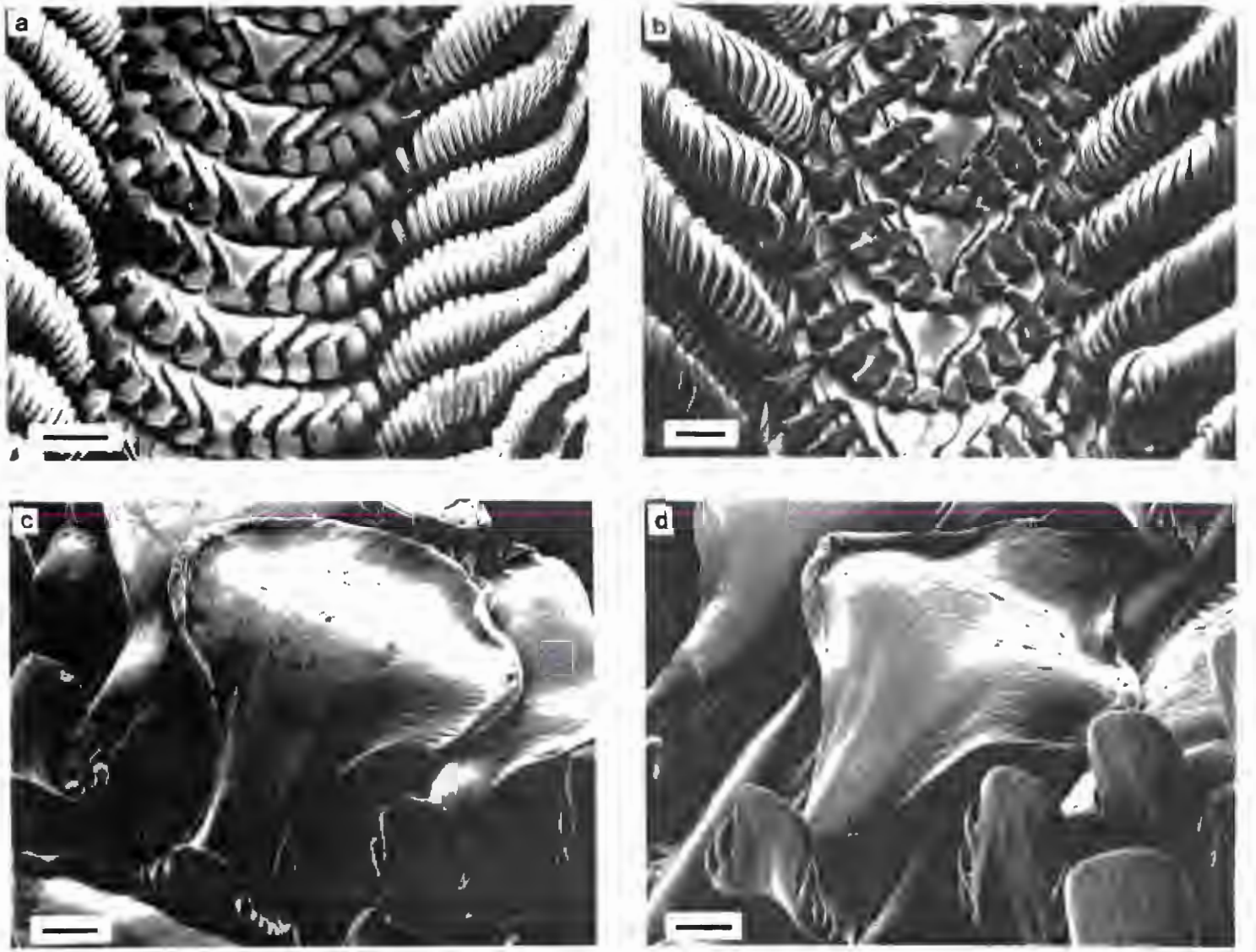
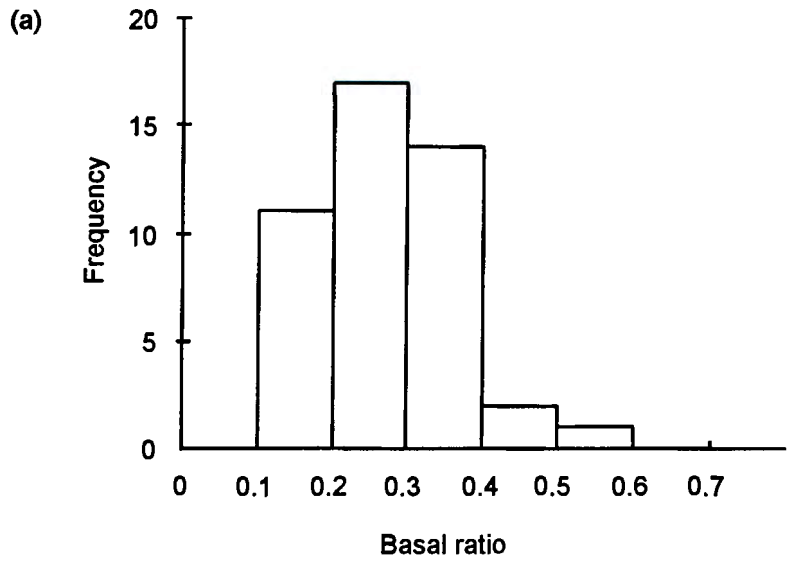


Plate 2. Scanning electron micrographs of the radulae of *Oxystele* species: (a) *O. impervia*, general view. Scale bar: 100 μ m. (b) *O. tigrina*, general view. Scale bar: 100 μ m. (c) *O. impervia*, central tooth. Scale bar: 20 μ m. (d) *O. variegata*, central tooth. Scale bar: 20 μ m.



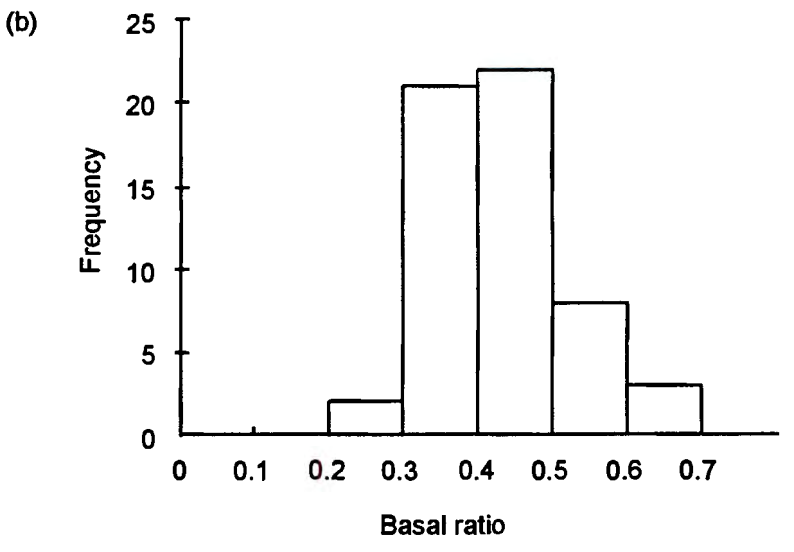


Figure 3. Frequency distribution of the basal ratios for (a) *O. impervia* and (b) *O. variegata*

Table 2. Variation in the extent of cusp indentation of the central radular tooth in *O. impervia* and *O. variegata* (shown as percentages). N = number of radulae examined.

	Slightly indented			Well indented		
Categories	1	2	3	4	5	6
O. impervia (N=53)	34	28	26	8	2	2
O. variegata (N=61)	0	21	20	28	15	16

O. impervia inhabits rocky shores, in the upper parts of the intertidal just below the *Littorina* zone. It is the highest *Oxystele* species on the South African shore.

In a recent survey around the South African coastline, *impervia* were always more abundant in the upper levels of the shore, and at no site were more than a few found in the lower levels of the shore. At sites exposed to strong wave action, very few *impervia* were found, and these were always in the upper zones (pers. comm.).

***Oxystele variegata* (Anton, 1838)**

Plate 1 (m-x)

Turbo variegatus Anton, 1838: 57.

Trochus indecorus Philippi, 1844-1846.

Trochus variegatus Philippi, 1851: 144 (no. 173), pl. 24 (figs 6, 15).

Oxystele sagittifera rufanensis Turton, 1932: pl. 44 (no.1281).

Oxystele variegata Richards, 1981: 67b.

Oxystele variegata Kilburn & Rippey, 1982: pl. 9-4 (two left shells).

Oxystele variegata Kensley & Penrith, 1972: pl. 13 (b - uppermost right shell).

Neotype No. SAM-A37549, from Dalebrook, Cape Peninsula, in the South African Museum, Cape Town (Plate 1m-o). A further specimen, SAM-A37550 from Dalebrook, is a paraneotype (p).

Diagnosis: Shell of medium size (shell diameter up to 31 mm, shell height up to 26 mm) with a rough texture. The pattern of the shell usually consists of two elements, ground colour and spiral bands.

Table 3. Mean and range of the basal ratio of the central tooth of the radula in *O. impervia* and *O. variegata* from each of the localities. N = number of radulae examined.

	O. impervia			*O. variegata*		
Locality	N	Mean	Range	N	Mean	Range
Brazil				4	0.463	0.412 - 0.552
Groen River	6	0.314	0.275 - 0.349	4	0.463	0.313 - 0.618
Camps Bay	3	0.334	0.250 - 0.419	5	0.489	0.389 - 0.581
Kommetjie	8	0.249	0.163 - 0.375	9	0.382	0.310 - 0.471
Scarborough	7	0.176	0.150 - 0.209	6	0.411	0.378 - 0.463
Buffelsbaai				5	0.423	0.357 - 0.636
Froggy Pond	3	0.241	0.220 - 0.279	5	0.453	0.400 - 0.545
Dalebrook	8	0.303	0.171 - 0.400	7	0.381	0.306 - 0.475
Kenton on Sea	6	0.301	0.196 - 0.586	5	0.432	0.333 - 0.571
Port Alfred	4	0.295	0.263 - 0.325	6	0.401	0.270 - 0.630

The ground colour, when present, consists of shades of greenish-grey to dull green (o,p,r). The spiral bands consist of alternating red and off-white maculations (r,u,w). In some shells, the greenish-grey ground colour seems to spread into the spiral bands, and covers much of the red maculations. The result is a hazy, greenish-grey to dull-green reticulate pattern, with occasional tinges of red (q). In other shells however, the greenish-grey ground colour is absent. In these shells the pattern consists of reddish diagonal (m,n), zigzag (x) or axial (u,v) lines, on an off-white background. Closer examination reveals how these lines are formed. The pattern actually consists entirely of spiral bands, composed of alternating reddish and whitish maculations, between which there is no ground colour at all. The red maculations of each spiral band interconnect with those above and beneath them, thus forming the pattern of diagonal, zigzag or axial lines.

The uppermost spiral band on each whorl, near the suture, (the "necklace"), usually differs from the others in that it is very broad, and its maculations are very stout and frequently squarish (o,r,s). The other spiral bands are narrower than the necklace. Two to three thin, horizontally-inclined reddish streaks sometimes appear just beneath the necklace (v).

In almost all shells, the red element can best be observed on the base of the shell, where it appears as maculations (or diagonal, zigzag or axial lines) on a greenish-grey or off-white ground colour. Very infrequently, the under part of the shell is all greenish-grey to black and the red component can only be seen with great difficulty.

In the central tooth of the radula (Plate 2d), the base is usually deep, so that the basal ratio is high (Fig. 3b). Our data are too scant to enable detailed statistical comparisons between *O. impervia* and *O. variegata* at each site, but it is noteworthy that at each site, *O. variegata* has a higher mean and observed range of the basal ratio than *O. impervia* (Table 3). When pooling all *O. variegata* as compared to *O. impervia*, the differences between them are highly significant ($Z=6.72$, $p<0.0001$, Mann-Whitney test).

The radula of *variegata* also differs from that of *impervia* in that the cusp of the central tooth is usually "well-indented" (59% of the individuals examined, as compared to 12% in *impervia*, see Table 2). In our two-category grouping, this difference between the two species is highly significant ($G=30.04$, $p<0.001$). In our six-category classification (where the lowest indentation category is absent in *variegata* but constitutes 34% in *impervia*, see Table 2), the difference is also significant ($G=43.00$, $p<0.001$). As with *O. impervia*, there is no suggestion of a geographic cline in the mean basal ratio of the radula.

Distribution and habitats: Our northernmost sample of *O. variegata* comes from Alexander Bay (680 km north of Cape Town) on the West Coast; our most eastern ones, from Kleinemonde, some 12 km east of Port Alfred (1000 km east of Cape Town) on the South-eastern Cape coast. *O. variegata* has recently been recorded (pers. comm.) at Dwesa (240 km east of Port Alfred) and also in relatively low numbers at Cape Vidal (250 km north of Durban). *O. variegata* thus spans a range of at least 2500 km. Day (1969) and Kilburn & Rippey (1982) recorded "*O. variegata* " from Luderitz (in Namibia) to Durban, but it is uncertain which of the two species these records refer to.

O. variegata inhabits rocky shores, in the middle parts of the intertidal zone. At every site in which we observed the two species, *variegata* was lower down the shore than *impervia*, although the two do overlap. A recent survey tends to support this trend. Where both *impervia* and *variegata* were found in reasonable numbers, *impervia* specimens were more abundant higher up the shore, whilst *variegata* specimens were found in greater numbers lower down on the shore. At some sites, however, very few specimens of *impervia* were found, and here *variegata* were found in relatively high numbers in the upper levels of the shore, as well as being found at lower levels (pers. comm.).

At Kenton on Sea, Port Alfred and Kleinemonde, a third *Oxystele* species, *O. tabularis*, was also found. It probably replaces *variegata* and *impervia* eastwards. At Dwesa and Cape Vidal large numbers of *tabularis* were recorded. At Kenton on Sea, *tabularis* was found mainly at a lower level than *variegata*, whereas at Kleinemonde it was found mainly above it. Thus we do not, as yet, have a clear-cut picture as to the zonation relationships between *tabularis* and *variegata*.

ELECTROPHORETIC ANALYSIS

The allele frequencies for the six loci and 18 populations are given in Appendix A. Most of these loci were highly polymorphic, with the number of alleles per locus ranging from three in *ARK* to 18 in *GPI*. Within any one population the maximum number of alleles per locus ranged from two in *ARK* to 11 in *AK*. However, a large proportion of the alleles were rare. Of the 18 alleles observed in *GPI*, only 4 had frequencies of more than 0.1. Likewise, only 4 out of 14 alleles in *AK* and *PGD*, and 6 out of 11 alleles in *LAP* had frequencies that exceed 0.1.

Out of 101 cases of polymorphism (using the 0.99 criterion) encountered in all loci and all populations, six showed significant ($p<0.05$) deviations from Hardy-Weinberg proportions. This represents about 6%, which is approximately what one could expect by chance alone. The deviations, all due to heterozygote deficits, occurred in two loci, three in each of *GL* and *ARK*.

A locus is diagnostic if individuals can be assigned to the correct species with a probability of 99% (Avise, 1975). Using this definition, two of the six loci examined here are taxonomically diagnostic. No alleles were shared by the two species at the *GL* locus. A total of seven alleles was observed, three of which occurred in all *impervia* populations (except one which had only two of the three alleles). The other four alleles, one of which was very rare and only found in two populations, were found only in the *variegata* populations.

The other locus found to be diagnostic was *PGD*. Only one allele was shared by both species. This allele occurred at a low frequency (< 0.03). A total of six individuals, from one *variegata* and four *impervia* populations, had this allele and all were heterozygotes with the alternative allele being the commonest one for the species. Three rare alleles (frequency < 0.026) were found, each in one individual (with each individual from a different population). In two of these cases, the individual was heterozygous, with the alternative allele being the commonest one

Table 4. Contingency table analysis of inter-sample and inter-specific allele frequency heterogeneity at four loci.

	Inter-sample											
	all samples			*O. impervia* samples			*O. variegata* samples			Inter-specific		
Locus	G	df	p	G	df	p	G	df	p	G	df	p
Ak	218.09	68	<0.001	50.26	28	<0.01	59.97	36	<0.01	107.86	4	<0.001
Gpi	433.73	68	<0.001	59.99	28	<0.001	16.87	36	NS	356.87	4	<0.001
Lap	708.6	68	<0.001	27.14	28	NS	49.47	36	NS	631.99	4	<0.001
Ark	325.39	17	<0.001	4.7	7	NS	248.53	9	<0.001	72.15	1	<0.001

for the species. In the third individual, the alternative allele, although rare, was only found in populations of the same species as this individual. In all the cases described above, the individual could still be assigned to the correct species with 99% certainty. Of the remaining ten alleles, six were found only in *impervia* populations. Two of these were common and occurred in all populations. The other four had lower frequencies, and occurred in seven, five, four and two out of eight populations respectively. Four alleles were found only in *variegata* populations, one of which occurred at a frequency of 0.895 or more in all ten populations. The other three had low frequencies (<0.065) and occurred in five, five and three out of ten populations respectively.

Prior to electrophoresis, the snails were separated into the two putative species by shell colour and pattern. It is noteworthy that out of a total of 423 individuals examined electrophoretically, only two shell-colour misidentifications were detected, by both *GL* and *PGD*.

Although not diagnostic, two of the other loci examined had at least one species-specific allele occurring at a frequency of 0.1 or greater. GPI^{20} (average frequency 0.289) was found in six out of the eight *impervia* populations, but in none of the *variegata* populations. This allele was absent in the two South-eastern Cape populations of *impervia*. Another three *GPI* alleles occurring at low frequencies (0.014 - 0.1) were found in at least three of the *impervia* populations but in no *variegata*, and another allele was found in at least three *variegata* populations, but in none of the *impervia*. LAP^{72} (average frequency 0.225) was also found in all of the *variegata* populations, but in none of the *impervia* ones. *LAP* also had two low frequency (0.025) alleles, both found in at least three *variegata* populations, but in no *impervia* ones.

Allele frequency heterogeneity was tested at the 1.25% significance level for the four non-diagnostic loci (*ARK*, *GPI*, *AK* and *LAP*). Due to the large number of rare alleles, the effective number of alleles was calculated (Ferguson, 1980). For *ARK*, two allele classes were used (the two faster-migrating alleles were pooled), and for the other three loci, five allele classes were used, the four most common alleles plus a pooled class of the remaining alleles. There was significant inter-sample heterogeneity at all four loci (Table 4). When only samples belonging to the same species

were compared (a total of eight separate comparisons), significant differences were found at the *GPI* and *AK* loci within the *impervia* populations, and at the *ARK* and *AK* loci for the *variegata* populations. The *GPI impervia* result can be explained by the presence of GPI^{20} in the one West Coast and five Cape Peninsula populations, and its absence in the two South-eastern Cape populations. Separate comparisons within the Cape Peninsula and South-eastern Cape populations found no significant differences in allele frequency, but there was a difference between the pooled Cape Peninsula populations and the pooled South-eastern Cape populations ($G=45.30$, $p<0.001$). A similar situation explains the *AK impervia* result, which is due to frequency differences at the AK^{86} and AK^{100} alleles between the South-eastern Cape populations and the remaining populations. The *AK variegata* difference can be explained by one South-eastern Cape population (Kenton on Sea) which differed in allele frequency (attributable to the AK^{109} and AK^{100} alleles) from the other *variegata* populations. No significant difference in the *variegata* populations was found when the Kenton on Sea population was excluded from the analysis. The difference between *variegata* populations at the *ARK* locus is due to the fact that the most common allele in both South-eastern Cape populations is different to that of the other *variegata* populations. The commonest allele in the South-eastern Cape populations (ARK^{140}) is fixed at Port Alfred, and occurs at a frequency of 0.975 at Kenton on Sea. The commonest allele for the remaining *variegata* populations (ARK^{100}), ranges in frequency from 0.850 to 1.00. In all the four cases where there were significant differences within a species (as described above), these were due to differences between one or both of the South-eastern Cape populations and the remaining populations. There were no significant differences between the two South-eastern Cape sites for all the loci tested within either species.

Interspecific comparisons were made using pooled South-eastern Cape populations and pooled West Coast and Cape Peninsula populations. In all these tests, there was a significant difference between the two species. All the populations of the same species were pooled for final interspecific comparisons, which were highly significant at the four loci (Table 4). Although there were some intraspecific differences, we can justify pooling the data, since our *a priori* intention was to look for differences between the two species.

Nei's genetic identity and distance results are summarized in Table 5. The mean identity within the *variegata* populations (0.917) is lower than that within the *impervia* populations (0.987). When the South-eastern Cape *variegata* populations are considered separately from the rest of the *variegata* populations, the mean identity between them is 0.775. It is this difference that is responsible for the lower overall identity value within this taxa. This result can be attributed mainly to the presence of a different common allele at the *ARK* locus in the two South-eastern Cape populations (as described above). This difference is also reflected in the dendrogram (Fig. 4) based on genetic differences. All of the *impervia* populations cluster together (including the South-eastern Cape ones in spite of allele frequency differences at two of the loci), as do the *variegata* populations excluding the two South-eastern Cape ones. The latter cluster together and are then joined with the rest of the *variegata*, before the two taxa are joined. The mean genetic distance between the two taxa is 0.584, whilst within either of the taxa $D=0.014$ and 0.094 for *impervia* and *variegata* respectively.

Combining the data for the *impervia* and *variegata* populations, Nei's genetic distances between all members of the *Oxystele* genus, were calculated. The relative amounts of differentiation between the five species are shown in Fig. 5. The *impervia* and *variegata* species form a cluster, which is linked to the other three species at a genetic distance which is much larger than the distance at which *impervia* and *variegata* are linked with each other.

Table 5. Summary of genetic identities (*I*) and distances (*D*) within and between populations of *O. impervia* and *O. variegata*. N = number of comparisons.

		Identity (*I*)		Distance (*D*)	
Comparison	N	Mean	Range	Mean	Range
impervia - impervia	35	0.987	0.955 - 1.000	0.014	0 - 0.046
variegata - variegata	45	0.917	0.712 - 1.000	0.094	0 - 0.339
variegata - variegata (no South east Cape)	28	0.995	0.966 - 1.000	0.005	0 - 0.034
impervia - variegata	153	0.641	0.140 - 1.000	0.584	0 - 1.965

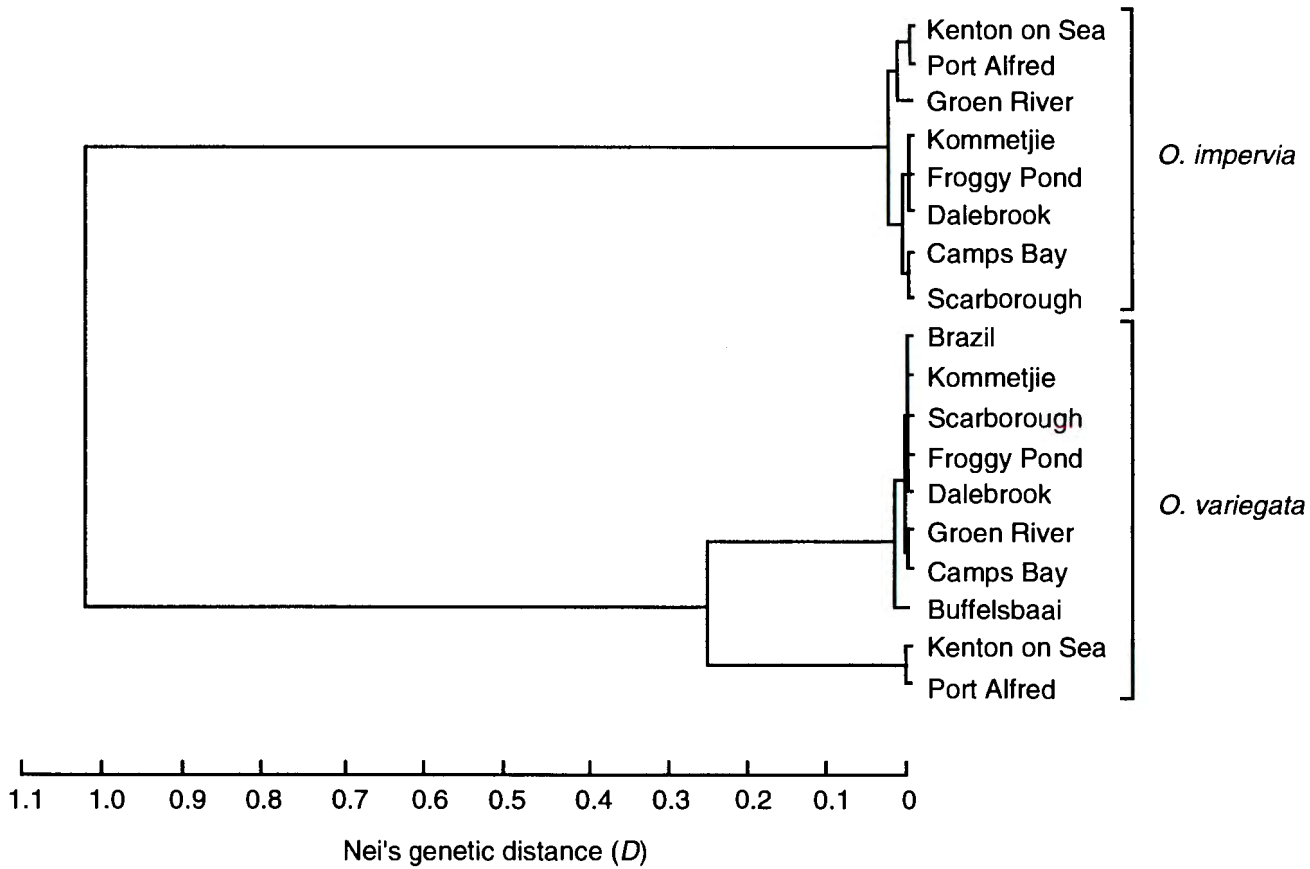


Figure 4. UPGMA cluster analysis showing the relationship between the 18 populations of *O. impervia* and *O. variegata* as determined by genetic distance.

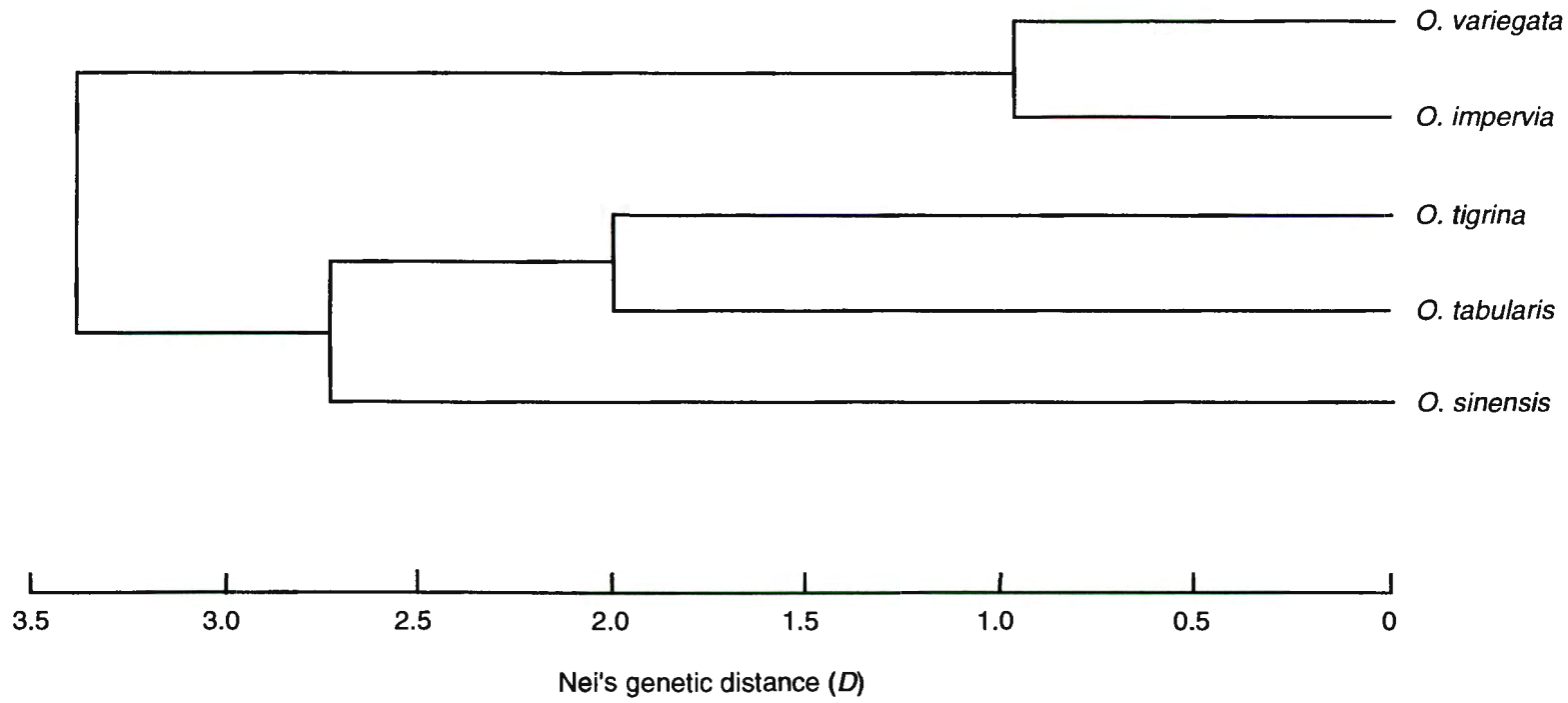


Figure 5. UPGMA cluster analysis indicating genetic differences between O. impervia and O. variegata relative to the other members in the genus.

KEY

(for all Southern African species of *Oxystele*)

(best used by examining shells beneath a dissecting microscope, or magnifying glass).

1a) shell always black to dark purple, sometimes with white specks 2

b) shell almost never black, usually variegated or striped (when shell is black, its underside has red stripes)..... 3

2a) inner lip crimson..... *O. sinensis*

b) inner lip white..... *O. tigrina*

3a) shell pattern of oblique maroon bands alternating with pinkish bands bearing a series of red and green axial lines; bands sometimes interupted spirally..... *O. tabularis*

b) shell pattern does not consist of oblique bands..... 4

4a) red maculations or lines on a ground colour that is either off-white, or greenish-grey to dull-green. These are best observed in the area just inside the insertion of the outer lip. Very infrequently, the underside of the shell is all-black..... *O. variegata*

b) dark red, orange, yellow or brown maculations (almost never lines), on a ground colour that is a either red, orange, yellow or brown..... *O. impervia*

DISCUSSION

TAXONOMIC REMARKS

The confusion between *variegata* and *impervia* dates back to the first half of the nineteenth century, when the two species were first described. Anton (1838) described the colour of *variegatus* as "gelb, weiss und braun reticulirt gebandert", a description which could fit either *variegata* or *impervia*. He described three varieties of *variegatus*. One, having a "Grund morgenroth", is probably an *impervia* (as described in this present paper). The two other varieties, "Grund weiss, mit dichten kirschenrothen ziczacstreifen" and "Grund gelblich weiss, braun gefleckt, basis roth punktirt" are clearly *variegata*, as here described. (Note that though the date printed on Anton's book is 1839, it had actually already appeared in 1838, - see Cernohorsky 1978). Anton's types are lost (Kilburn, pers. comm.).

Menke (1843) described the colour of *impervius* as being "cinerea vel rubida, lineis ex alb articulatis confertis fasciaque maculari infra suturam cincta", a description that could fit either of the two species described here. Menke's types are also lost (Kilburn, pers. comm.).

In 1848, Krauss listed *variegatus* as a mere variety of *impervius*. This lumping by Krauss was questioned by Philippi (1851), who listed the two as separate and described and illustrated them with such excellent clarity and accuracy that we follow him, in this present paper, in the nomenclature of the two species.

Later, however, Sowerby (1892) listed *variegatus* and *sagittiferus* as synonyms of *impervius*, and Turton (1932) listed *impervia* and *variegata* as varieties of *sagittifera*. Barnard (1963) listed *impervius* and the *sagittifera* described by many previous authors as synonyms of *variegata*, and this opinion has since been accepted by Day (1969), Kensley & Penrith (1972), Richards (1981) and Kilburn & Rippey (1982). Almost every single sample of "*O. variegata*" examined by us in the Cape

Town Museum mollusc collection was found to contain a mixture of the two species. With hindsight it is not difficult to see why *impervia* and *variegata* were considered as a single species, as they are both very variable in shell colour, and both share the presence of a necklace.

Trochus indecorus was described from South Africa by Philippi (1844-1846) as having dark red or black dash bands, on a ground colour that is dirty green or grey, a description that well fits our own description of *O. variegata*, whereby we consider it a synonym.

Turton (1932) described several *Oxystele* taxa from Port Alfred. His description of *O. sagittifera rufanensis*, as having a uniform dark colour, agrees with the pattern of *O. variegata* that we collected from Port Alfred, whereby we consider it a synonym of the latter. His *O. sagittifera perdix* (Koch) is a synonym of *impervia*, as could be judged from his photograph of it. His *O. carinata*, described as having a light orange brown shell, corresponds to the *O. impervia* that we collected from that locality; it was probably a juvenile. His *O. farquhari*, in which the shell "is a reddish orange with several spirals of yellow rectangular dots" could well be an *impervia*. However, the small size of his specimen (5 mm) and the poor quality of the photograph he published prevent us from reaching of definite conclusions without examining the material.

The precise relation between *O. variegata* and *O. sagittifera* Lamarck is unclear. Philippi (1851) noted that Lamarck's description is insufficient, that Delessert's figure of this species is probably of a different exemplar and that in the collection of the Museum National d'Histoire Naturelle, *variegatus* was under the name *sagittiferus*. He concluded that *sagittiferus* is a doubtful species. Nickles (1950) figured an *O. sagittifera* from Mauritania which lacks the typical necklace of *variegata*. Examination of excellent quality colour slides of the holotype of *sagittiferus* in the Museum National d'Histoire Naturelle collection, labelled in Lamarck's handwriting, shows that there is no necklace; that the ground colour is yellowish; and that the spiral bands consist of alternating black and white maculations. Also, a moderately pronounced tooth (as is typical of the genus *Monodonta*) is found on the columella. None of these traits are found in either *impervia* or *variegata*. We therefore consider *sagittiferus* as a different species.

Kensley & Penrith (1972) suggested that *Oxystele fulgurata* Philippi, 1848 of southern Angola, is a synonym of what they named *variegata*. We cannot comment on their suggestion, for lack of comparative material from Angola (and from Guinea, from where *O. fulgurata* was originally described).

COLOUR VARIATION

Shell colour variation in both *O. impervia* and *O. variegata* is an interplay between two factors: ground colour and maculated spiral bands (the necklace is the uppermost band). By expanding the maculated spiral bands in breadth, at the expense of the ground colour, an original shell pattern of spiral bands may be transformed into one of axial or diagonal stripes. These two factors appear to be basic units in the evolution of colour, not only within the genus *Oxystele*, but also in a very wide array of topshells. The interplay between them can be used to describe the shell patterns of such distantly related species as the trochids *Calliostoma consor, C. punctatum, Cantharidus bellulus, C. opalus, C. ramburi, Clanculus miniatus, C. pharaonius, Gibbula capensis, G. umbilicalis, G. cineraria, Oxystele tigrina, Thalotia conica, Tectus conus, T. niloticus, Trochus erythraeus, T. kochi, T. virgata*,and *Umbunium giganteum*; the turbinids *Turbo cidaris, T. militaris, T. petholatus*, and *T. reevei*; and the phasianellids *Tricolia speciosa*, and *Phasianella australia*. (See colour photographs of these species in Dance, 1974; Richards, 1981 and Lindner, 1978). They may thus be basic units in the evolution of colour within the Trochacea.

A central issue in prosobranch shell colours is the extent to which they are genetically inherited. The Australian trochid *Austrocochlea constricta* shows a variable pattern of shell banding and the concentration of its main shell pigment, uroporphyrin I, is primarily governed by environmental factors, increasing as the chlorophyll content of the substratum increases (Creese & Underwood, 1976; Underwood & Creese, 1976). It is as yet unknown to what extent the shell colours of *O. impervia* and *O. variegata* are environmentally induced. Colour consists of hue and of

shade, and we tentatively believe that the hues of these species may be genetically determined while the shades may be environmental.

O. impervia and *O. variegata* exhibit an immense variety of shell colours and pattern, often within a single population. Both species live on highly varied backgrounds, of multicoloured rocks that are sometimes further coloured by algal encrustations. Recent investigations, upon limpets mismatched to their background and exposed to fish and bird predators, suggest that selection by visually-hunting predators can be remarkably strong in the rocky intertidal (Mercurio *et al.*, 1985). In a previous study, in England, it was possible to single out one rock formation, Red Sandstone, and to show that the frequency of red shells of *Littorina rudis* and *L. nigrolineata* is associated with the presence of this formation (Heller, 1975). In South Africa we could not find a similar homogeneously coloured rock. In their natural habitat, most *O. impervia* and *O. variegata* shells seem cryptic to the human eye: The shape of the shell is broken up by the maculated spiral bands, which vary considerably from one zone of the shell to another (the band on the crest of the whorl is often more pronounced than the others); and these bands overlay a ground colour that varies considerably throughout shell growth. The combination of these two elements contributes towards shell crypsis. The diversity of colour variation in *O. impervia* and *O. variegata* may prevent their major visual predators from forming a search image for more than a fraction of the population at any one time.

Though it may seem readily understandable why colour variation has evolved within these two species, the situation may actually be more complex. On the very same shores that support populations of *O. impervia* and *O. variegata* with highly diverse colours, in the lower intertidal, two other *Oxystele* species are frequently found that are not polymorphic: *O. tigrina* which is monomorphic black with a few white specks, and *O. sinensis* which is monomorphic purplish-black with a crimson inner lip. It could perhaps be argued that their black monomorphism is due to past accidental genetic constraints that prevent them from developing colour variation. However, it is noteworthy that a remarkably similar situation is also found in England, involving a different genus, *Littorina*. The upper intertidal is occupied by *L. rudis/saxatilis* and *L. nigrolineata*, which are highly

variable in colour, whereas the lower intertidal is occupied by *L. littorea*, which is all-black (Heller, 1975). This parallel between *Littorina* and *Oxystele*, in two completely different parts of the world, suggests that the occurence of black shells in *O. tigrina* and *O. sinensis* is not due to accidental genetic constraints. Possibly, forces of selection differ considerably in different zones of the intertidal. In some cases they may favour variably-coloured shells in the upper zones and monomorphic black ones further down.

DIFFERENCES IN THE RADULA

The radulae of both species resemble the typical trochid pattern (Fretter & Graham, 1962), suggesting that the snails feed upon whatever they encounter on the rock surface, exerting little choice in food material. McQuaid (1983) examined the gut contents and faeces of what he named "*variegata*" and found large numbers of diatoms, together with organic matter that may be of macrophytic origin. Wynberg (1985) found that a large proportion of the gut contents of several *Oxystele* species consisted of unidentifiable organic matter and fragments of the soft chlorophyte *Ulva*. She also found a high concentration of detritus and sand in the guts, implying that the species all feed in a similar and relatively non-selective manner. Ozinsky (1985) excluded what she named "*variegata*" from caged areas at the top of the shore, and found that these ungrazed rocks exhibited considerable growth of *Porphyra capensis*, the chlorophyte *Ulva* sp., *Hildebrandia* sp. and *Calothrix* sp. She concluded that the *Oxystele* populations on the top of the shore (most probably *impervia*) are supported by macroalgae which exhibit virtually zero standing crop but a high productivity through sporeling growth (which becomes apparent when the topshells are excluded).

O. tigrina possesses lateral and central teeth that are longer than those of *impervia* and *variegata*. These differences suggest that somewhat different foods (and/or somewhat different feeding mechanisms) may be available to the various co-occurring *Oxystele* species, perhaps as a result of the slightly different zones that they occupy on the shore. The longer teeth may suggest

that *O. tigrina*, lower down the shore, tends to use long sweeping movements to collect its food, whereas *impervia* and *variegata*, higher up, tend to use short brushing movements.

O. tigrina possesses a well-indented central tooth, broadly similar to that of *O. variegata*, whereas *O. impervia*, which is the uppermost species on the shore, has a slightly-indented one. Weak indentation may present a further trend in a shift from sweeping to brushing, in the higher zones of the rocky intertidal shore. The functional significance of the difference between *O. impervia* and *O. variegata* in the basal ratio of the central tooth is unknown.

ELECTROPHORETIC VARIATION

At those sites where the two putative species co-occur, the most frequent allele in each species was different in 34 out of a possible 48 cases (eight sites and six loci). The failure to find a single individual possessing both the most frequent allele of *impervia* and also that of *variegata* indicates reduced or no gene flow between the two, implying reproductive isolation. The existence of two diagnostic loci, of significant heterogeneity in allele frequency (between the two species) at the other loci, and of species-specific alleles at two of these loci, further supports the conclusion that they are indeed two separate species.

There have been a number of other studies on gastropods in which the presence of significant allele frequency differences, and diagnostic loci, have indicated the existence of two species occurring in sympatry (Chambers, 1978; Murphy, 1978; Mastro *et al.*, 1982; Janson, 1985; Ward & Janson, 1985). Studies (on both invertebrates and vertebrates) have indicated that between a quarter and a half of the loci examined in closely related species are almost completely distinct, whereas in conspecific populations, there is often a high degree of similarity between the populations over the entire range of the species (Avise, 1975).

In contrast to the large differences found between the two species, within either of the species there was very little genetic differentiation, even between populations at opposite ends of the range examined. There were, however, a few exceptions, all concerning the two South-eastern Cape sites. Within the *variegata* populations, a different common allele occurred at the *ARK* locus at both sites, as well as significant heterogeneity in allele frequency at the *AK* locus at one of these sites - Kenton on Sea. Within the *impervia* populations, there was significant heterogeneity in allele frequency at the *AK* and *GPI* loci at both sites when compared to the other sites. A number of studies have shown that there is genetic uniformity over the geographic range of gastropods which have a pelagic larval dispersal stage (Gooch et al., 1972; Berger, 1973; Hoagland, 1985). It is generally hypothesised that extensive gene flow reduces the effects of localized selection (Soule, 1976; Crisp,1978; Berger, 1983). However, Burton (1983) pointed out that not all species with planktonic larvae show uniform distribution of alleles over their range. He further warned against basing conclusions on population structure on the basis of examining a few loci. Hedgecock (1986) considered differentiation between conspecific populations to be the rule rather than the exception, despite larval dispersal. This differentiation can arise either because of biological or physical barriers to larval dispersal, or from differential survival of recruits, or from differential mating success. *O. impervia* and *O. variegata* both have a pelagic larval stage, as do all the Trochidae, and one may expect a certain amount of mixing of the gene pool to occur. Although it appears that gene flow is important in maintaining the genetic uniformity found among *impervia* and *variegata*, it is not necessarily the only factor involved. There does appear to be some localized divergence at the two South-eastern Cape populations of both *impervia* and *variegata*. Without further study, we cannot be sure of the reason for this localized divergence.

Our calculations of Nei's genetic similarity (*I*) and distance (*D*) are based on only a few loci. As such they will produce biased results and should not be used for comparison with other values in the literature. If anything, our *D* values are over-estimates, whilst our *I* values are under-estimates (since only loci showing differences between the two species were examined). The *I* values within the two species, even as under-estimates, are well within the range of what has been found for conspecific populations (about 93% of the *I* values within species exceed 0.9, Thorpe,

1982). Nei's values can, however, be used as a relative measure of the degree of differentation between the populations examined in this study.The mean genetic distance between the two species (0.584) is an order of magnitude greater than it is within either of the two species (0.014 for *impervia* and 0.094 for *variegata*). Although we cannot use Nei's *D* to make an accurate taxonomic assessment of specific status, the dendogram (Fig. 4) clearly shows two distinct groupings.

Fig. 5 shows the position of *O. impervia* and *O. variegata* in relation to the other three South African members in the genus. Although the values for these other species are based on only a few individuals, they do indicate that *O. impervia* and *O. variegata* are separate but closely related species.

The coupling of morphometric with electrophoretic data has proved to be very valuable in solving many systematic problems (e.g. Mastro *et al.*, 1982; Ward & Janson, 1985; Hoagland & Davis, 1987). Biochemical data showing genetic isolation are regarded as being sufficient to conclude the existence of two species when they occur sympatrically (Hoagland, 1984). This is what we have found in this study; in addition, the congruency between morphological and biochemical data sets strengthens the conclusions considerably.

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Appendix A. Allele frequencies of *O. variegata* and *O. impervia* populations from 10 localities. N = number of individuals examined.

	O. variegata										*O. impervia*							
Locus allele	Brazil	Groen River	Camps Bay	Komm-etjie	Scar-borough	Buffels-baai	Froggy Pond	Dale-brook	Kenton on Sea	Port Alfred	Groen River	Camps Bay	Komm-etjie	Scar-borough	Froggy Pond	Dale-brook	Kenton on Sea	Port Alfred
Ak																		
130	-	-	-	-	-	0.038	-	-	-	-	0.017	-	-	-	-	-	-	-
120	-	-	-	-	-	-	0.050	-	-	-	-	-	-	0.025	-	-	-	-
117	-	0.020	0.075	0.025	-	0.026	-	0.027	0.025	0.067	0.017	-	0.025	-	-	-	-	0.050
109	0.600	0.521	0.400	0.500	0.368	0.256	0.350	0.405	0.150	0.400	0.117	0.125	0.100	0.075	0.200	0.189	0.158	0.100
105	0.050	0.020	-	-	0.132	0.128	0.125	0.041	0.100	-	0.067	-	-	0.100	-	-	-	0.025
100	0.300	0.313	0.400	0.375	0.421	0.397	0.375	0.446	0.625	0.433	0.333	0.600	0.575	0.525	0.550	0.541	0.395	0.300
95	0.025	-	0.025	0.050	-	0.013	0.050	-	-	-	0.033	-	-	0.075	-	-	-	0.100
91	-	0.063	0.025	-	-	0.077	-	0.027	-	0.067	0.100	0.075	0.050	0.025	-	0.014	0.026	-
86	0.025	0.063	0.050	-	0.053	0.051	-	0.054	0.100	0.033	0.217	0.200	0.075	0.125	0.150	0.176	0.368	0.400
83	-	-	-	0.025	-	-	0.025	-	-	-	0.033	-	0.075	-	0.075	-	-	-
77	-	-	0.025	-	0.026	0.013	-	-	-	-	0.050	-	0.025	0.025	-	0.027	-	-
73	-	-	-	0.025	-	-	0.025	-	-	-	-	-	0.075	-	-	0.041	0.026	0.025
65	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.025	0.014	0.026	-
53	-	-	-	-	-	-	-	-	-	-	0.017	-	-	0.025	-	-	-	-
N	20	24	20	20	19	39	20	37	20	15	30	20	20	20	20	37	19	20
Ark																		
180	-	-	-	0.025	-	-	-	-	-	-	0.017	-	0.050	-	-	-	-	-
140	-	-	0.100	0.125	0.125	0.118	0.050	0.125	0.975	1.000	-	0.050	-	0.025	-	-	0.079	0.050
100	1.000	1.000	0.900	0.850	0.875	0.882	0.950	0.875	0.025	-	0.983	0.950	0.950	0.975	1.000	1.000	0.921	0.950
N	20	24	20	20	20	38	20	36	20	15	30	20	20	20	20	37	19	20

(cont.)

Appendix (continued)

	O. variegata										*O. impervia*							
Locus allele	Brazil	Groen River	Camps Bay	Komm-etjie	Scar-borough	Buffels-baai	Froggy Pond	Dale-brook	Kenton on Sea	Port Alfred	Groen River	Camps Bay	Komm-etjie	Scar-borough	Froggy Pond	Dale-brook	Kenton on Sea	Port Alfred
Gpi																		
185	-	-	-	-	-	0.013	-	-	-	-	-	-	-	-	-	-	-	-
155	-	0.021	-	0.075	-	-	-	-	0.025	-	-	-	-	-	-	-	-	-
132	0.300	0.167	0.225	0.150	0.125	0.218	0.275	0.257	0.150	0.167	0.033	-	0.025	-	0.050	0.041	-	0.025
125	-	-	-	-	-	-	-	0.027	-	-	-	-	-	-	-	-	-	-
115	-	-	-	-	-	0.013	-	-	-	0.033	-	-	-	-	-	-	-	-
106	0.025	-	0.025	-	-	-	-	-	-	-	-	0.025	-	-	-	-	-	-
100	0.675	0.729	0.675	0.675	0.800	0.718	0.675	0.635	0.775	0.733	0.350	0.150	0.175	0.200	0.225	0.176	0.526	0.450
94	-	-	0.025	-	-	-	-	-	-	-	-	0.025	-	-	-	-	-	-
72	-	-	-	-	-	-	-	-	-	-	0.033	0.050	0.025	-	-	0.014	-	-
65	-	0.042	0.050	0.075	-	0.038	0.050	0.068	0.025	0.067	0.300	0.325	0.400	0.325	0.350	0.351	0.395	0.475
50	-	0.042	-	0.025	0.075	-	-	0.014	0.025	-	0.017	-	0.025	0.025	-	-	0.079	0.050
33	-	-	-	-	-	-	-	-	-	-	-	0.050	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-	0.233	0.300	0.275	0.300	0.300	0.324	-	-
-20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.014	-	-
-30	-	-	-	-	-	-	-	-	-	-	0.017	0.075	0.050	0.100	-	0.081	-	-
-40	-	-	-	-	-	-	-	-	-	-	0.017	-	-	-	-	-	-	-
-64	-	-	-	-	-	-	-	-	-	-	-	-	0.025	0.050	0.050	-	-	-
-95	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.025	-	-	-
N	20	24	20	20	20	39	20	37	20	15	30	20	20	20	20	37	19	20
Lap																		
113	-	-	-	-	-	-	-	-	0.025	-	0.034	-	-	0.025	-	-	0.026	-
108	-	-	-	0.050	-	-	-	-	-	-	-	0.100	0.050	-	-	0.015	0.079	-
104	0.025	-	-	-	-	-	-	-	-	0.036	0.069	-	0.025	-	-	-	0.026	0.025
100	-	-	0.029	-	0.050	0.026	-	0.017	0.025	-	0.776	0.750	0.700	0.950	0.889	0.742	0.763	0.750
94	0.050	0.021	-	-	0.200	0.105	-	0.052	0.025	0.036	0.052	0.025	0.050	-	-	0.091	0.053	0.075
90	0.100	0.104	0.059	0.100	0.050	0.132	0.050	0.155	0.050	0.286	0.052	0.050	0.025	-	-	0.106	0.026	0.025
85	0.600	0.583	0.676	0.700	0.575	0.474	0.500	0.534	0.575	0.429	-	0.075	0.150	0.025	-	0.045	0.026	0.125
80	-	-	0.029	-	-	0.026	-	-	-	0.036	0.017	-	-	-	0.111	-	-	-
72	0.225	0.292	0.206	0.125	0.125	0.184	0.425	0.241	0.250	0.179	-	-	-	-	-	-	-	-
67	-	-	-	-	-	0.026	0.025	-	0.025	-	-	-	-	-	-	-	-	-
61	-	-	-	0.025	-	0.026	-	-	0.025	-	-	-	-	-	-	-	-	-
N	20	24	17	20	20	38	20	29	20	14	29	20	20	20	18	33	19	20

(cont.)

Appendix (continued)

Locus allele	*O. variegata*										*O. impervia*							
	Brazil	Groen River	Camps Bay	Komm-etjie	Scar-borough	Buffels-baai	Froggy Pond	Dale-brook	Kenton on Sea	Port Alfred	Groen River	Camps Bay	Komm-etjie	Scar-borough	Froggy Pond	Dale-brook	Kenton on Sea	Port Alfred
GI																		
102	-	-	-	-	-	-	-	-	-	-	0.036	0.125	-	0.350	0.050	0.095	0.132	0.100
100	-	-	-	-	-	-	-	-	-	-	0.804	0.750	0.925	0.525	0.875	0.878	0.816	0.850
98	-	-	-	-	-	-	-	-	-	-	0.161	0.125	0.075	0.125	0.075	0.027	0.053	0.050
91	0.225	0.044	0.275	0.350	0.350	0.141	0.325	0.324	0.225	0.433	-	-	-	-	-	-	-	-
88	0.700	0.956	0.675	0.650	0.650	0.526	0.650	0.622	0.750	0.533	-	-	-	-	-	-	-	-
84	0.050	-	0.025	-	-	0.333	0.025	0.054	0.025	0.033	-	-	-	-	-	-	-	-
78	0.025	-	0.025	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N	20	23	20	20	20	39	20	37	20	15	28	20	20	20	20	37	19	20
Pgd																		
280	-	-	-	-	0.025	0.013	0.026	0.027	-	0.033	-	-	-	-	-	-	-	-
270	-	-	-	-	-	0.013	-	-	-	-	-	-	-	-	-	-	-	-
255	0.025	-	0.025	-	-	0.013	-	-	-	-	-	-	-	-	-	-	-	-
240	-	-	-	-	-	0.013	-	-	-	-	-	-	-	-	-	-	-	-
220	0.975	0.937	0.925	1.000	0.975	0.923	0.895	0.932	1.000	0.967	-	-	-	-	-	-	-	-
210	-	-	0.025	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
170	-	-	-	-	-	-	0.026	-	-	-	0.017	0.026	-	-	-	0.027	0.026	-
150	-	0.063	0.025	-	-	0.026	0.053	0.041	-	-	-	-	-	-	-	-	-	-
140	-	-	-	-	-	-	-	-	-	-	0.017	0.211	0.175	0.167	0.200	0.284	0.290	0.175
100	-	-	-	-	-	-	-	-	-	-	0.917	0.737	0.675	0.694	0.650	0.581	0.605	0.675
90	-	-	-	-	-	-	-	-	-	-	0.033	-	-	0.028	-	0.014	-	0.100
60	-	-	-	-	-	-	-	-	-	-	0.017	-	0.050	0.056	0.125	0.068	0.079	0.050
45	-	-	-	-	-	-	-	-	-	-	-	0.026	0.050	0.056	0.025	0.014	-	-
25	-	-	-	-	-	-	-	-	-	-	-	-	0.050	-	-	0.014	-	-
N	20	24	20	20	20	39	19	37	20	15	30	19	20	18	20	37	19	20

Chapter 7

Synthesis

INTRODUCTION

Many taxonomic problems have arisen in marine molluscs that have been identified and classified based on morphometric characters only, in particular the shell. This is not surprising in view of the well known phenomenon of shell plasticity in the face of environmental influences, which can result in considerable variation within species (e.g. Phillips *et al.*, 1973; Kitching, 1976; Crothers, 1983; Palmer, 1990; Boulding *et al.*, 1993). The species of *Burnupena* are no exception. Members of this ecologically important genus are among the commonest and most abundant species inhabiting the Southern African coastline, yet they have lead to taxonomic confusions for over a century (e.g. Kiener, 1834; Tryon, 1881; Stephenson, 1948; Kilburn & Rippey, 1982), and revisions of the genus by Orr (1956) and Barnard (1959) based on shell and radular characters have failed to fully resolve the problems.

Enzyme electrophoresis has been successfully employed in a large number of studies of marine molluscs (e.g. Ward & Warwick, 1980; Mastro *et al.*, 1982; Hoagland, 1984; Ward & Janson, 1985; Palmer *et al.*, 1990; McDonald *et al.*, 1991; Boulding *et al.*, 1993) to solve systematic problems. In this study, electrophoretic analyses have allowed resolution (and detection) of species that would have remained impossible on purely morphological grounds.

INTRASPECIFIC VARIATION IN *BURNUPENA*

Burnupena species do not have a pelagic larval stage. They lay eggs on the substratum, and the young snails hatch as crawlaways (Bokenham *et al.*, 1938). Although little is known about the extent of dispersal by adults, it has been suggested that they are probably rather sedentary (Bokenham *et al.*, 1938). Given this, one might anticipate that levels of intraspecific variation, both

morphological and electrophoretic, will be high within the species of *Burnupena*, and that conspecific populations in close proximity should be less differentiated than those that are well separated geographically.

The analyses of morphological variation have shown that the degree variation is relatively large within all of the species, a common finding in studies of other marine gastropods (e.g. Phillips *et al.*, 1973; Janson & Sundberg, 1983; Thomas & Himmelman, 1988; Boulding, 1990; Palmer *et al.*, 1990). Within species, the form and colour of the shell can be influenced by local environmental conditions, giving rise to differences between local populations. However, broad geographical trends were revealed. Differences between populations in different regions were greater than the differences between populations in the same region, and populations in one region were more likely to be confused with populations in a neighbouring region than with populations in non-adjacent regions. Despite the high levels of intraspecific morphological variation, cluster analyses revealed that most of the populations of individual species tended to cluster together. For the two species sampled from at least three regions (*B. cincta* and *B. lagenaria*), the Western Overlap populations were shown to be more similar to the South Coast populations, than to the West Coast populations. This finding supports the zoogeographic regions distinguished by Emanuel *et al.* (1992), who combined the Western Overlap and South Coast regions within their Warm Temperate South Coast province.

One of the previously recognised species, *B. limbosa*, was reduced to a subspecies of *B. cincta* on the basis that they are largely geographically separated and are morphologically distinct only in their ribbing, which appears to be environmentally influenced, possibly by different temperature regimes. Results from other species supported this hypothesis, since populations on the West Coast tended to have smooth shells, whilst populations in the Western Overlap and South Coast were ribbed. Furthermore, the genetic distances between the populations of *B. limbosa* and *B. cincta* were very small, and well within the range expected for conspecifics (Thorpe, 1982).

Like the shell, the radula was shown to be highly variable within most of the *Burnupena* species, the exception being the new species, *B.* sp. B. The variability within species was such that the species could not be distinguished from one another. Moreover, radulae were also found to be variable within individuals, and a number of abnormalities were detected which might be environmentally induced, further rendering the radula useless as a means of separating species (although it did distinguish the genus *Burnupena* from related genera).

The electrophoretic analyses revealed that, similar to the morphological variation, the level of variation within each of the species was relatively high. At least 60% of the total genetic variation within each of the species was attributable to differences between individuals within populations, with differentiation between populations comprising a much smaller proportion.

The genetic distances between conspecific populations were mostly very low, and well within the range expected for conspecifics (Thorpe, 1982). However, for two populations (Durban *B. lagenaria* and Port Elizabeth *B. pubescens*), that were well separated geographically from their conspecifics, the genetic distances were at least twice as high as most of the remaining conspecific distances. However, even these were still within the range expected for conspecifics, and there was nothing to suggest that these particular populations were anything other than well differentiated allopatric populations. Most of the conspecific populations within a region clustered together, and, as with the cluster analysis of the morphological variables, all of the populations of the same species clustered together. However, for *B. cincta* and *B. lagenaria*, the Western Overlap populations were found to be genetically more similar to West Coast populations, opposite to the pattern found with the morphological variables.

INTERSPECIFIC DIFFERENTIATION IN *BURNUPENA*

The discovery of high levels of intraspecific variation within the species of *Burnupena*, in both the morphological and electrophoretic data, reiterates the importance of determining such variation before attempting to assess levels of differentiation between closely related species.

Relative to the levels of intraspecific variation, the differences between the species based on multivariate analysis of shell morphology were not high. However, distinctions between the species could be discerned, although some combinations of species remained blurred. In particular, many of the difficulties of identification occurred between *B. cincta* and *B. lagenaria*, and between various species on the West Coast. The multivariate analyses grouped the species into those that were most similar morphologically. For the most part, it was between these same groups of species that difficulties in identification have arisen in the past. However, despite the large overlap in variability between the species within these groups, only a small proportion of the individuals were incorrectly identified. The overall conclusion from the analyses of shell morphology was that whilst many of the species could be distinguished from most of the other species, there would always be a few populations and/or individuals which would be difficult to identify with certainty.

The descriptions of each of the species in Chapter 4 include details of the shell and aperture colour, features which could not be used in the multivariate analyses. These, together with particular characters (or suites of characters), were used to distinguish between morphologically similar species, and permitted correct identification of the majority of the individuals in the genus. *B. cincta cincta* and *B. lagenaria* remain one of the most confusing pairs of taxa: although the extreme forms are distinctive, phenotypic intermediates are not uncommon. However, these two species can usually, but not always, be distinguished by the colour of the aperture and of the shell.

Examinations of radulae revealed that whilst some differences between the species were detected, these were usually not consistent, either within or between species, although *B.*

catarrhacta showed a few minor differences from the other species. Kool (1987) argued that similarities between radulae are most likely to result from common descent. Genetic results demonstrated that *B. catarrhacta* is clearly very distinct from the other species, so if Kool is correct, then one might expect to find more differences between the radula of *B. catarrhacta* and those of other *Burnupena* species. However, as discussed above, the radula was on the whole deemed to have little value at the specific level, although it is clearly an important character at the generic level.

Unlike the morphological analyses, the results of the electrophoretic analyses revealed that the species were generally well differentiated genetically. When all species were considered simultaneously, almost twice as much of the differentiation between all populations, was due to differences between species, rather than differences between the populations within species. Not all of the species were equally differentiated from all other populations however, and in this respect, two results were of interest. The first was that *B. catarrhacta* was very well differentiated from the other species. Almost 70% of the loci were diagnostic for this species, and the genetic distances were such that *B. catarrhacta* could be regarded as a different genus. The second point of interest, and at the opposite end of the scale to *B. catarrhacta*, was the very low levels of differentiation between *B. cincta* and *B. lagenaria* relative to the other species. Individuals belonging to these two species could not always be unambiguously separated genetically - a fact reflected in the biochemical key constructed to distinguish the species.

Despite the low levels of differentiation between *B. cincta* and *B. lagenaria*, they, together with all of the other species, were genetically distinct. All of the populations of each species clustered together before linking to other species. Furthermore, between all pairs of sympatric populations, reproductive isolation was total in 95% of cases, as evidenced by the lack of shared alleles at one or more loci. Even in the remaining 5% of cases, reproductive isolation was highly likely, judging by the absence of overlap of genotypes.

The phylogenetic hypotheses produced from both the morphological and allozyme data sets supported a number of findings. Firstly, all of the evolutionary trees indicate that *B. cincta* and *B. limbosa* are very closely related, lending support for my decision to reduce them to subspecies. Secondly, all of the trees show that *B. lagenaria*, whilst distinct, is closely related to *B. cincta*. Thirdly, *B. catarrhacta* was shown to be the most primitive species and a sister to all of the other species. The congruence of these particular results derived from the two independent data sets increases confidence in them.

The hypotheses based on the cladistic analysis of the allozyme data were considered to be a more accurate reflection of the true phylogeny of *Burnupena*. Apart from the results noted above, the allozyme cladograms also indicated that *B. papyracea* and *B. pubescens* were closely related, and that the group consisting of *B. papyracea*, *B. pubescens*, *B. lagenaria*, *B. cincta cincta* and *B. c. limbosa* was monophyletic and thus shared a common ancestor.

COMPARISON OF MORPHOLOGICAL AND ELECTROPHORETIC ANALYSES

The phenotype of the shell is a product of its genotype and the influence of the environment on the genotype. If the shell phenotype, as represented by the characters used in the multivariate analysis (Chapter 1), has a large genetic component, then a correlation between morphological and electrophoretic divergence might be expected. This would suggest that shell characters could be useful for determining evolutionary relationships (Dillon & Davis, 1980). If, on the other hand, shell morphology is largely influenced by the environment, then no such correlation can be expected. Rather, one might expect to find sympatric species which are genetically distinct, but morphologically similar, and allopatric conspecific populations which are genetically similar but which may be morphologically similar or different depending upon differences in the environment.

Euclidean distances (calculated for the cluster analyses in Chapter 1) between all pairwise comparisons, were used as a measure of morphological divergence between all of the populations of *Burnupena*. Nei's genetic distance between all pairs of populations, represents genetic divergence. The relationship between these two measures of divergence is shown in Fig. 1. The Pearson correlation coefficient between morphological and genetic divergence for all pairwise comparisons was 0.33 ($N=351$, $p<0.01$), and that for the subset consisting of all the pairwise comparisons between the congeneric species was 0.20 ($N=286$, $p<0.01$). These were both significant, but the correlation between the subset consisting only of the pairwise comparisons between conspecific populations was not significant ($r=0.06$, $N=65$). This suggests that within species, local environmental conditions affect shell morphology, which is reflected in the high level of intraspecific variation that was detected, but that morphological divergence between species may have a greater genetic component. There was no trend indicating that pairs of sympatric species might be more similar morphologically than pairs of allopatric conspecifics.

The distribution of the data points along the x-axis in Fig. 1 was expected, based on the results of the electrophoretic analyses. The genetic distances between the conspecific populations were the smallest, whilst the bimodal distribution of the distances between species reflects the large distances between the populations of *B. catarrhacta* and other species. These results conform to genetic distance estimates found between molluscs, where the majority of distances between conspecifics were less than 0.10, whilst most congeneric distances were usually between 0.20 and 0.60 (Woodruff et *al.*, 1988). The distribution of the data points along the y-axis on the other hand, reflect the high levels of morphological variation within species, as well as the wide range of morphological differentiation between congeneric populations.

Two of the species, *B. cincta* and *B. lagenaria*, were sampled from a number of localities in at least three regions. For both species, the morphometric analyses revealed that the Western Overlap populations were more similar to the South Coast populations (seen in the cluster and discriminant analyses). However, with the electrophoretic data, the Western Overlap populations

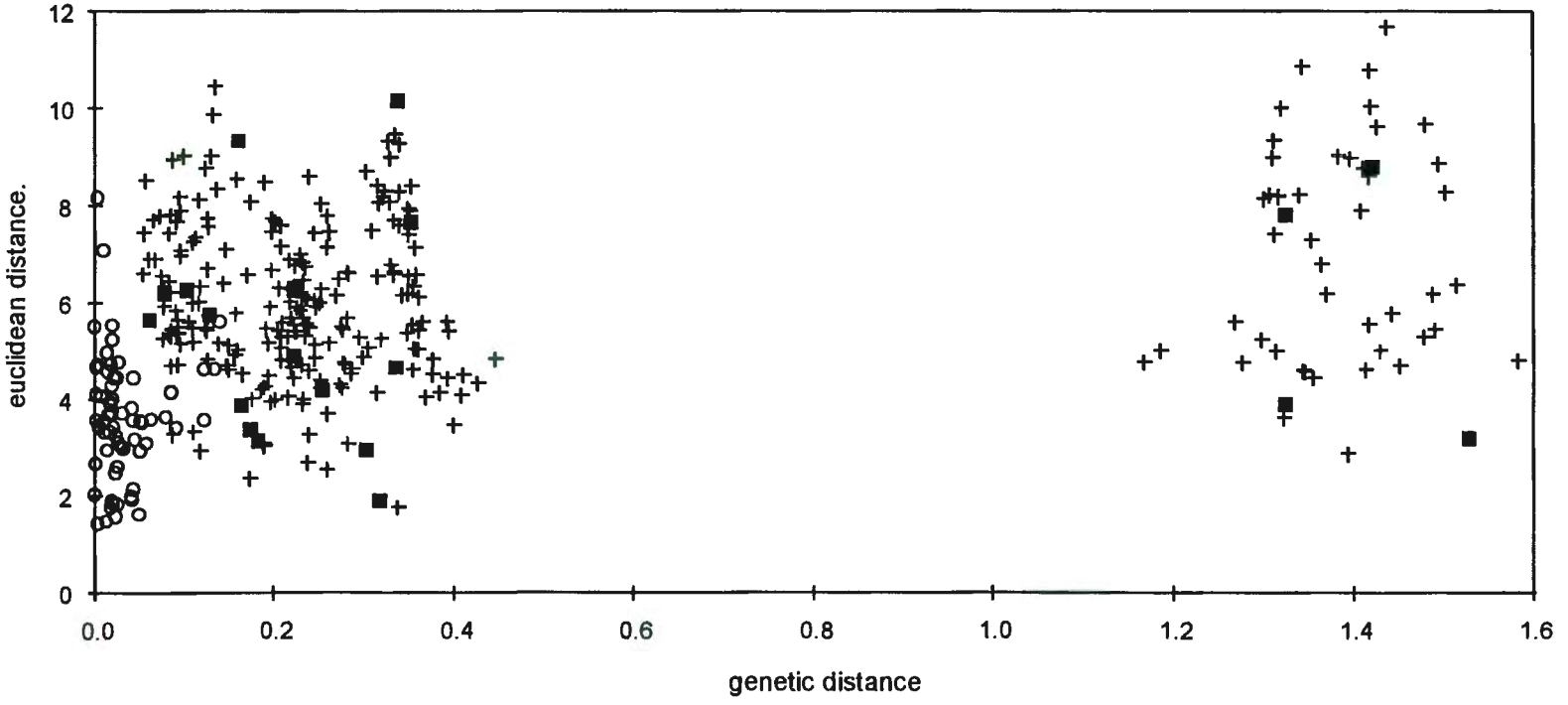

Figure 1. Relationship between morphological and genetic distances between all populations of *Burnupena*. The open circles (o) indicate pairs of conspecific populations; the solid squares (■) indicate pairs of sympatric congeners; the pluses (+) indicate pairs of allopatric congeners.

were more similar to the West Coast populations in both species. In their study of the zoogeographic regions around the coast of southern Africa, Emanuel *et al.* (1992) combined the Western Overlap and South Coast regions based on the distribution patterns of 2000 species of invertebrates. As suggested above, local environmental conditions can substantially affect shell morphology within species, implying that electrophoretic similarities might more accurately represent regional affinities. However, since there was a strong correlation between genetic and geographic distances ($p<0.001$ for both species, Chapter 3), the greater similarity between the Western Overlap and West Coast populations for the electrophoretic data may simply reflect the smaller geographic distances between the populations sampled in these two regions, than between the populations sampled in the Western Overlap and South Coast regions.

USE OF BIOCHEMICAL TECHNIQUES IN DETECTING CRYPTIC SPECIES

Protein electrophoresis has been used for the detection of a number of morphologically cryptic species (e.g. Chambers, 1978; Mastro *et al.*, 1982; Ward & Janson, 1985; Staub *et al.*, 1990; Thorpe & Solé-Cava, 1994). In some cases, the discovery has been unexpected, whilst in others, electrophoresis has been used to confirm the existence of suspected cryptic species.

During my study of the genetic variability in *Burnupena*, a new species, which I have referred to as *B.* sp. B, was discovered. On morphological grounds this species was initially identified as belonging to either *B. pubescens* or *B. papyracea*, but the results of the enzyme electrophoresis indicated that it is clearly genetically distinct from both, having a number of diagnostic loci and no gene flow between sympatric populations at at least two loci. Although there are small but consistent differences between its shells and those of other species (Chapter 4), these were not initially obvious, and clear and consistent differences were only sought after it became evident that a new species was at hand. Without genetic reasons to suspect the presence of a cryptic

species, it is highly unlikely that *B.* sp. B would have been detected. This species is morphologically very similar to *B. pubescens*, and to a lesser extent *B. papyracea*, and like both of these species, it is covered by a bryozoan. It differs only in a few shell characters which could have easily been regarded as part of the natural variability of either *B. pubescens* or *B. papyracea*.

In the case of *Oxystele variegata*, the presence of a cryptic species was suspected after initial examination of these snails in the Cape Peninsula revealed several colour differences between some of the shells. Electrophoresis was used to confirm our suspicions. In this study only a few loci were examined, but they unambiguously revealed a lack of gene flow between sympatric populations of the two putative taxa, providing clear evidence that two species, *O. variegata* and *O. impervia*, were present. Differences between these two species were also apparent in the radula.

RELATIONSHIPS BETWEEN POPULATION DIFFERENTIATION AND LARVAL DISPERSAL

A number of studies have shown that there appears to be a relationship between larval dispersal and genetic differentiation between populations. Species with direct development appear to exhibit greater geographic differentiation than do species that have a pelagic larval dispersal stage (e.g. Gooch *et al.*, 1972; Berger, 1973; Crisp, 1978; Hoagland, 1985; Janson, 1987; Day & Bayne, 1988; Day, 1990; Liu *et al.*, 1991). It is generally hypothesised that extensive gene flow reduces the effects of localized selection leading to increased genetic homogeneity (Soule, 1976; Crisp, 1978; Berger, 1983). However, a number of authors (Burton, 1983; Hedgecock, 1986) have cautioned against inferring levels of gene flow from apparent dispersal capabilities, pointing out that whilst marine invertebrates without pelagic larvae do tend to show greater population differentiation than species having planktonic larvae, substantial differentiation has been observed in species that appear to have high dispersal capabilities. Hedgecock (1986) considered differentiation between conspecific populations to be the rule rather than the exception, despite larval dispersal. He noted

that differentiation could arise from a number of factors, such as barriers to larval dispersal, differential survival of recruits, or differential mating success. In a study of the phylogeny of members of the genus *Littorina*, Reid (1990) found that, of populations of *Littorina* that had migrated to the northern Atlantic from the Pacific, one population with planktotrophic development, *L. littorea*, had not speciated, while a second population which lacked planktonic larval forms had undergone rapid speciation producing six species. Reid commented that this agreed with the recognized potential of those species lacking planktonic development to achieve geographic isolation and allopatric speciation.

As noted above, *Burnupena* species do not have a pelagic larval stage and the adults are probably rather sedentary. Although low relative to the levels of variation within populations, substantial differentiation between populations in each of the species was detected, indicating constraints on gene flow between certain populations. In Chapter 3 it was found that, in the four species that could be tested, and for all species together, there was a significant correlation ($p<0.01$) between genetic and geographic distances (Fig. 2a). This pattern is consistent with Wright's (1943) isolation-by-distance model of the genetic structure of species, whereby individuals within species exchange genes with those individuals that are closest to them geographically. The relatively large amounts of genetic differentiation observed between geographically well separated populations were, therefore, not unexpected, and are consistent with the lack of a larval dispersal phase.

The members of the genus *Oxystele* have a pelagic larval stage, and therefore it is possible to compare the levels of population differentiation between *Burnupena* and *Oxystele* in the light of their different modes of reproduction. Only a few loci were examined in the study of *O. variegata* and *O. impervia*, and therefore any conclusions drawn must be treated with caution. However, it is possible to assess levels of population differentiation within each of these two species and comment on their hypothesised higher potential for greater gene flow, and hence increased genetic uniformity, relative to the situation found in *Burnupena*. The relationship between genetic and geographic distances for *O. variegata* and *O. impervia* are shown in Figs 2b and 2c respectively.

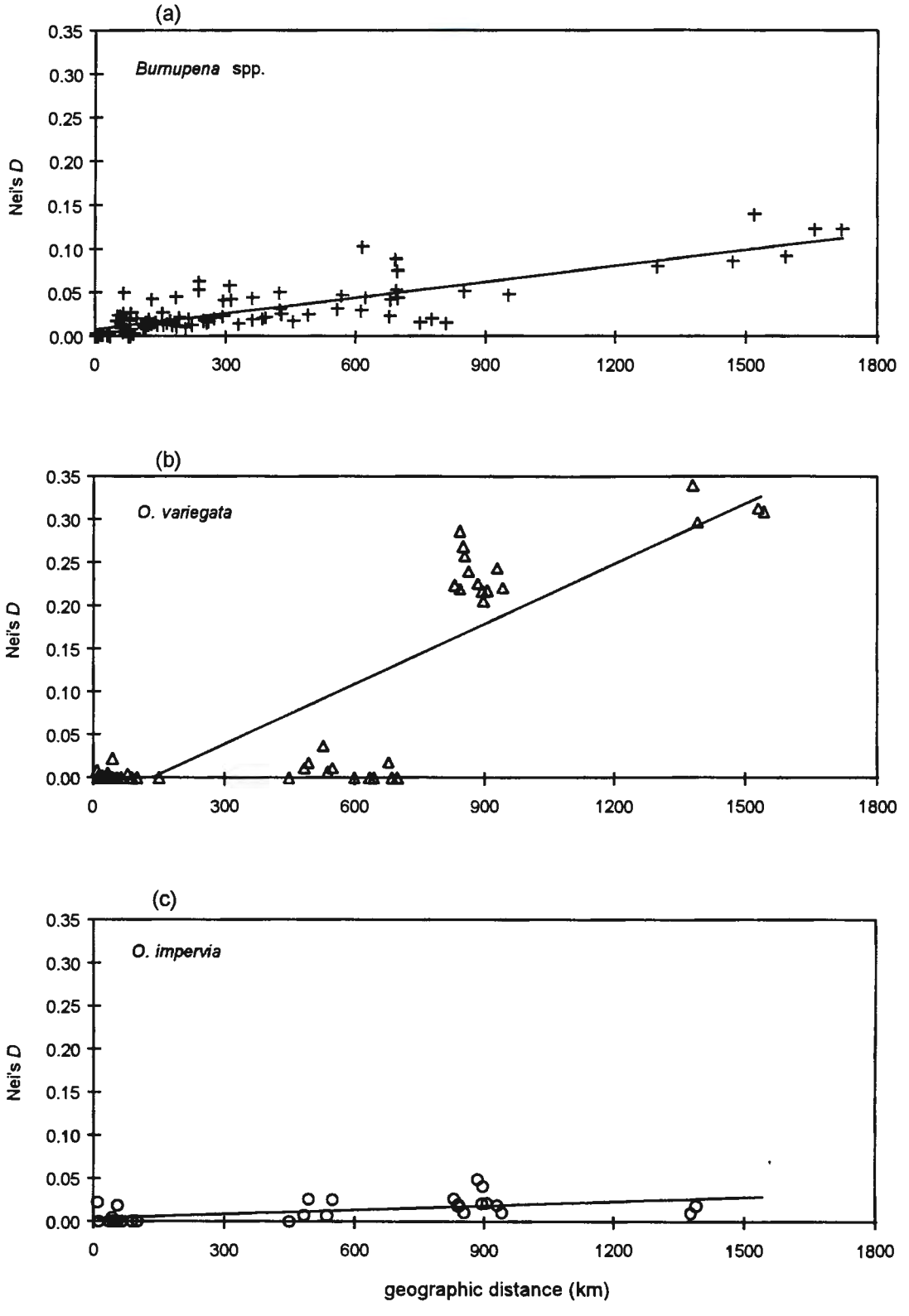

Figure 2. Nei's genetic distance (*D*) as a function of geographic distance between (a) conspecific populations of *Burnupena*, (b) populations of *O. variegata*, and (c) populations of *O. impervia*. The regression lines are shown. All axes have the same scale for ease of comparison.

The results show that the two species of *Oxystele* exhibit clearly different trends, with an apparently strong relationship between genetic and geographic distance for populations of *O. variegata*, but little to no such relationship for populations of *O. impervia*. It must be remembered that the genetic distances between the populations are likely to be overestimates (Chapter 6), and that the observed relationship for *O. variegata* may not be as strong as indicated. However, even at much reduced genetic distances, it is likely that a positive relationship would exist. The results for *O. impervia* will not be affected. The results for *O. variegata* are interesting (Fig. 2b), for although there is an overall positive relationship between genetic and geographic distances, this appears not to be the case for all pairs of populations. The data are grouped into four clusters, with the two groups in the upper right section representing distances between the two South Coast populations and the remaining populations. The group with low genetic distances but separated by distances of around 600km represent pairs of populations from the Cape Peninsula area with those from the West Coast. Populations in this latter group show a similar pattern to the populations of *O. impervia* (Fig. 2c). Excluding the two South Coast populations of *O. variegata*, these results would suggest that these two species of *Oxystele* exhibit a greater degree of genetic homogeneity than do the species of *Burnupena*. However, the two South Coast populations of *O. variegata* clearly do not fit this pattern, and are more similar to the *Burnupena* species, or exhibit even greater genetic distance between populations. However, why there should be differentiation of the *O. variegata* but not the *O. impervia* South Coast populations are not clear. The fact remains that the two species of *Oxystele*, which are so similar in life style, occupy near-identical habitats and have pelagic larvae, have contrasting patterns of genetic distance between populations. Furthermore, the linear regressions for Nei's *D* on distance shows that *O. variegata* has (on average) greater genetic differentiation between populations than the *Burnupena* spp., while *O. impervia* has less differentiation than *Burnupena*.

CONCLUSIONS

Multivariate analyses have proved useful in that they allow the information contained in a number of morphometric variables to be summarized such that they can differentiate between the groups better than can individual morphometric variables (Reyment *et al.*, 1984). They also allow levels of variation, both intra- and interspecific, to be more easily assessed. Although the multivariate analyses of morphometric variables produced groups of species of *Burnupena* that were most similar, the results derived from different methods indicated that the groupings were not robust, and depended upon the method chosen. Thus, caution should be exercised when choosing a technique, and preferably more than one multivariate approach should be utilized.

The application of enzyme electrophoresis to the taxonomic problems of both *Burnupena* and *Oxystele*, has clearly helped resolve a number of problems. In the case of *Burnupena*, an understanding of the levels of intraspecific genetic variation was required before conclusions could be made regarding differences between the species. Issues such as levels of variation both within populations and within species, amount of differentiation between species, and evolutionary relationships among the species, were determined from the results of the electrophoretic analyses. Different questions were posed by the *Oxystele* study, requiring a slightly different approach to the application of the enzyme electrophoresis. If two sympatric taxa are shown to be fixed for different alleles at a particular locus, then the two taxa must be reproductively isolated (Buth, 1984). Thus, by examining a number of sympatric populations along the range of *"O. variegata"*, it was possible to detect the presence of a cryptic species using only a few loci.

This study was undertaken initially because of problems with the identification of some of the species belonging to *Burnupena*. At the onset, six species of *Burnupena* were recognised, namely, *B. catarrhacta*, *B. cincta*, *B. lagenaria*, *B. limbosa*, *B. papyracea* and *B. pubescens*. Detailed morphological and electrophoretic studies have lead me to conclude that of these six species, five are valid, but that *B. limbosa* should be reduced to a subspecies of *B. cincta*. Furthermore, these

studies revealed the presence of two new species. Kilburn and Rippey (1982) described the genus *Burnupena* as a "headache". A few individuals and/or populations will always remain a headache to identify on morphological grounds, but even these problem cases can almost always be separated electrophoretically. The species, as outlined above, are usually morphologically and virtually always electrophoretically distinct. Resolution of the boundaries between species would have been impossible without the joint application of morphological and genetic approaches.

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